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14816

The Action of Gramine.

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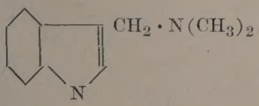
In the course of investigating Swedish barley, chemically and genetically, von Euler and Hellström[1] discovered a crystalline alkaloid, named gramine, in 2 chlorophyll-deficient mutants. Subsequently, Orechoff and Norkina[2] succeeded in isolating an alkaloid, called donaxine, from an Asiatic reed (botanically known as *Arundo donax* L.), which later proved to be identical with gramine.[3,4] Wieland and Hsing[5] showed that gramine conforms to the structure of 3-dimethylaminomethylindole, as follows:



Gramine was investigated pharmacologically by Supniewski and Serafenówna,[6] who showed that it caused a weak parasympathomimetic action. Raymond-Hamet[7] observed that small doses of gramine raised, but large doses lowered, the blood pressure in dogs.

In our laboratory, studies have been conducted with indole derivatives, both natural and synthetic. To explore this field further, gramine was tested pharmacologically in animals. Our sample was generously supplied by Dr. Richard H. F. Manske, National Research

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[1] von Euler, H., and Hellström, H., *Z. f. physiol. Chem.*, 1933, **217**, 23.

[2] Orechoff, A., and Norkina, S., *Ber. d. deut. Chem. Gesellsch.*, 1936, **68B**, 436.

[3] von Euler, H., Erdtman, H., and Hellström, H., *Ibid.*, 1936, **69**, 743.

[4] Brandt, K., von Euler, H., Hellström, H., and Löfgren, N., *Z. f. physiol. Chem.*, 1935, **235**, 37.

[5] Wieland, T., and Hsing, C. Y., *Liebig's Ann. d. Chem.*, 1936, **526**, 188.

[6] Supniewski, J. W., and Serafinówna, M., *Bull. internat. Acad. polon. d. sc. et d. lett., Cl. méd.*, 1937, 479.

[7] Raymond-Hamet, *C. R. Soc. de biol.*, 1937, **126**, 859; 1939, **130**, 1218.

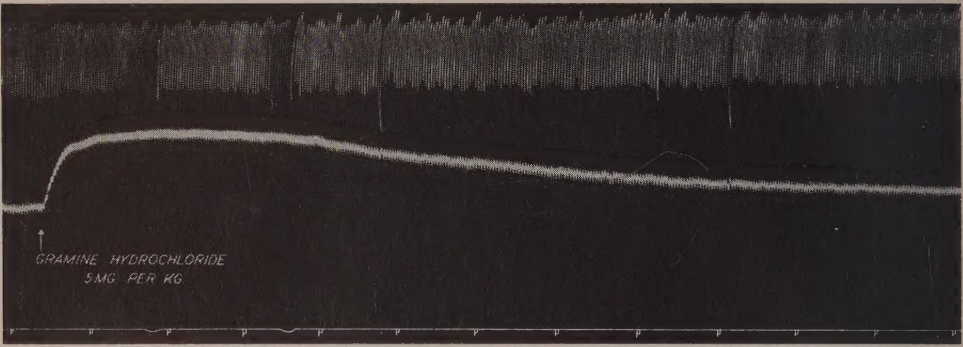

Fig. 1.
Action of Gramine on Blood Pressure and Respiration.
Cat, female, weighing 1.91 kg, was anesthetized by intraperitoneal injection of "Sodium Amytal" (Sodium Iso-amyl Ethyl Barbiturate, Lilly), 80 mg per kg. Records from above down are: respiration, carotid blood pressure, and baseline with time marks in minutes. Gramine HCl in 1% solution was injected rapidly into the femoral vein. The blood pressure rose from 100 to a maximum of 160 mm Hg, lasting for more than 12 minutes.

Council, Ottawa, Canada, and was originally isolated from *Arundo donax* L. The alkaloid was converted into the hydrochloride, in our laboratory, by dissolving the solid base in a solution of an equimolecular amount of hydrochloric acid. After evaporation under a fan, the residue was taken up in absolute ethanol. Upon the addition of anhydrous ether, the hydrochloride crystallized out with ease. The whole procedure must be carried out quickly, otherwise decomposition as evidenced by rapid discoloration takes place. This salt apparently had not been prepared previously. Gramine hydrochloride is freely soluble in water and melts at 190.5-191.0°C (corrected) with decomposition. Analysis for nitrogen satisfies the required composition, $C_{11}H_{14}N_2 \cdot HCl$ (Calculated: N 13.3%; found: N 13.1%). For pharmacologic experiments, fresh solutions were prepared each day.

1. *Action on Circulation.* In 6 anesthetized cats, 17 observations were made on the blood pressure and respiration following the intravenous injection of gramine HCl. Doses ranging from 1-20 mg per kg uniformly caused a sustained rise of arterial blood pressure, an example of which is shown in Fig. 1. No changes in respiratory amplitude took place. Amounts of 30-40 mg lowered the blood pressure, followed by a secondary rise. There was a decrease in amplitude of respiration immediately after injection with prompt return to normal. This change was most likely secondary to the initial fall of blood pressure.

It was also noted that epinephrine following the larger doses of gramine HCl, 30-40 mg per kg, became less effective in raising blood pressure in the same animal. Thus, there is an apparent inhibition of epinephrine response by gramine. In no instance, however, did a reversal of blood pressure occur in contrast with epinephrine-ergotoxine antagonism. The same phenomenon was previously observed by Raymond-Hamet.[7]

Eight tests on the blood vessels of 3 frogs were made by Trendelenburg's method of perfusion. There was a slight decrease of perfusing rate with a concentration of 1:20,000, an evidence of vasoconstriction. The effect became less noticeable with a solution of 1:40,000, but disappeared with a concentration of 1:80,000.

Eleven observations were made on 5 frogs' hearts perfused by the method of Howell and Cooke.[8] A solution of 1:40,000 produced a slight decrease in amplitude and rate of ventricular contractions, but one of 1:5,000 resulted in a very marked decrease in amplitude which was easily reversed by returning to Ringer's solution. A typical record is shown in Fig. 2. Dilute solutions such as 1:100,000

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[8] Howell, W. H., and Cooke, E., *J. Physiol.*, 1893, **14**, 198.

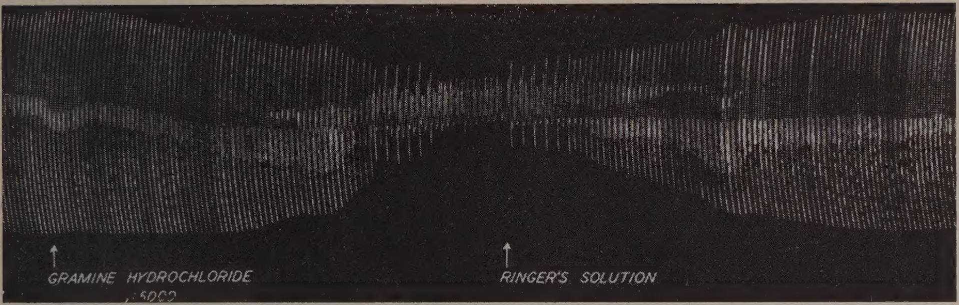

FIG. 2.
Action of Gramine on Frog's Heart.
Leopard frog, female, weighing 84 g, was perfused through inferior vena cava. The depressing action of gramine HCl is obvious, which is reversible by substitution with Ringer's solution.

were ineffective. No distinct stimulation of cardiac contractions was noted with any of the concentrations.

2. *Action on Smooth Muscle Organs.* When solutions of gramine HCl, as high as 4%, were instilled in the rabbit's eye, no change in the diameter of the pupil took place.

Ten tests were made with gramine HCl on strips of isolated rabbits' small intestines immersed in Tyrode's solution warmed to 38°C. Inhibition of peristaltic movements promptly occurred. The effect was definite with concentrations varying from 1:20,000-1:50,000. In most instances, a brief, slight stimulation appeared before inhibition took place. Recovery from inhibition was rapid and complete. Although epinephrine also inhibited peristaltic movements of the same intestines, its response was definitely reduced by a previous application of gramine HCl. The 2 substances are again antagonistic to each other, and certainly not synergistic.

The isolated uteri of 3 species of animals were studied with gramine HCl—rabbits, guinea pigs, and Syrian hamsters (*Cricetus auratus*). Thirty-five assays were made with 12 strips of rabbits' uteri immersed in Tyrode's solution. A dilution of 1:100,000 produced stimulation of contractions as shown in Fig. 3, A. The guinea pig's uterus immersed in Locke-Ringer's solution required larger doses, such as 1:20,000, as shown in Fig. 3, B. Seventeen tests on 5 strips were carried out in that species. The hamster's uterus immersed in Locke-Ringer's solution, like that of the rat, has rhythmic contractions normally. Upon application of gramine HCl, spastic contractions resulted as indicated in Fig. 3, C. Concentrations of 1:20,000-1:10,000 were most suitable.

As is well known, epinephrine also contracts the isolated rabbit's uterus. Our results indicate that when the latter was first treated with gramine HCl, its response to epinephrine was diminished. Again, there is evidence of antagonism between epinephrine and gramine.

3. *Effect on Blood Sugar.* Majority of pressor substances have a hyperglycemic action when administered intravenously. It was thus interesting to ascertain whether or not gramine might have a similar action. Six rabbits were injected intravenously, 2 each, with doses of 5, 10, and 20 mg of gramine HCl per kg of body weight. Their blood sugar was determined at 30-minute intervals according to the procedure of Hagedorn and Jensen.[9] No significant changes in blood sugar were found.

4. *Toxicity.* The median lethal doses (LD_{50}) of gramine HCl were determined in albino mice and rats by intravenous injection. The results are summarized in Table I. Mice, having an $LD_{50} \pm$ S.E. of 44.6 ± 1.4 mg per kg, are more susceptible than rats, which have an $LD_{50} \pm$ S.E. of 62.9 ± 2.7 mg per kg. A series of tonic convulsions with an apparent increase of respiratory rate, protrusion of eye-

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[9] Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46.

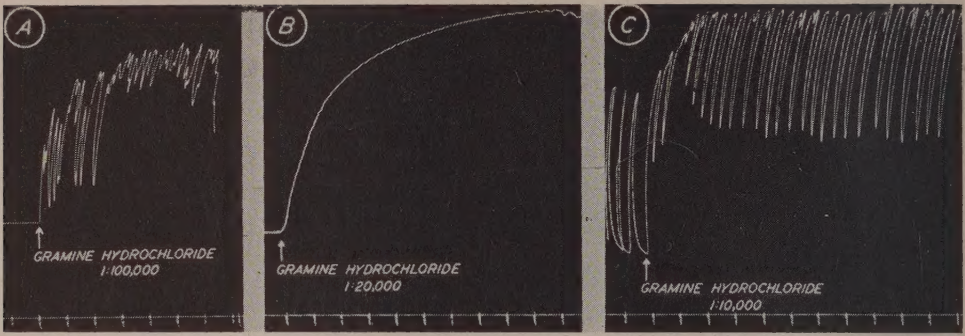

FIG. 3.
Action of Gramine on Isolated Uteri.
A. Rabbit's uterus immersed in Tyrode's solution at 38°C.
B. Guinea pig's uterus immersed in Locke-Ringer's solution at 38°C.
C. Hamster's uterus immersed in Locke-Ringer's solution at 38°C.

balls, and suggestive cyanosis occurred following the administration of lethal and near-lethal doses. With mice, there was a persistent erection of the tail—a phenomenon very similar to that following the administration of morphine.

Six frogs were injected in the lymph sac with gramine HCl in doses ranging from 0.2-1.0 mg per g. These are relatively large doses if expressed on a kilogram basis. All the animals showed depression and prostration, with stretching of hind legs and sagging of front legs. When placed on their backs, they made no attempt to right themselves, even upon the application of mechanical stimulation. This is in contrast with warm-blooded animals, as represented by mice and rats which exhibited evidence of central stimulation following the intravenous injection of gramine HCl.

TABLE I.
Acute Toxicity of Gramine HCl.

Animal	Dose, mg/kg	No. died / No. used	$LD_{50} \pm$ S.E. mg/kg
Mice	36.5	1/10	44.6 ± 1.4
	40.0	4/10	
	45.0	3/10	
	50.0	8/10	
	60.0	10/10	
Rats	50.0	0/5	62.9 ± 2.7
	56.0	4/10	
	62.0	5/10	
	70.0	6/10	
	80.0	9/10	

Summary. 1. Gramine hydrochloride raises blood pressure in anesthetized cats in small doses but lowers it, with a secondary rise, in doses of 30-40 mg per kg. 2. In appropriate concentrations, gramine HCl by perfusion in frogs causes slight constriction of peripheral blood vessels. 3. Gramine HCl when perfused into the inferior vena cava of frogs decreases the amplitude and rate of heart contractions. 4. Gramine HCl inhibits, frequently preceded by a brief stimulation, peristaltic movements of isolated rabbits' small intestines. 5. Gramine HCl contracts the isolated uteri of rabbits, guinea pigs, and Syrian hamsters. 6. Gramine HCl reduces the effect of epinephrine on blood pressure, intestinal movements, and uterine (isolated) contractions, but causes no reversal. 7. Gramine HCl in doses of 5-20 mg per kg does not alter the concentrations of blood sugar in rabbits. 8. The toxicity of gramine HCl has been determined by intravenous injection in both mice and rats, the latter being less susceptible.

The authors are indebted to Messrs. R. C. Anderson, John C. Hanson, and Harold M. Worth for their invaluable assistance in these experiments.

14817 P

A Characterization of a Urinary Fraction Capable of Producing Myeloid Metaplasia in Guinea Pigs*

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Heinle, Wearn, Weir, and Rose[1] reported myeloid hyperplasia and metaplasia in guinea pigs following the injection of extracts and adsorbates of human urine. The majority of potent preparations had been obtained from patients with chronic myeloid leukemia. The highest yield of active material appeared to result if the urine was processed by the benzoic acid adsorption method of Katzman and Doisy.[2] We have now further purified this fraction and studied some of its chemical and physical properties as well as some of the quantitative aspects of its effects on guinea pigs.

The benzoic acid adsorbate, prepared from urine of patients with chronic myeloid leukemia, was freed of adsorbent by extraction with acetone. The insoluble residue was leached with a sufficient volume of M/2 boric acid buffer (pH 8.5) to produce an extract of a pH greater than 7. The solution was acidified with acetic acid (pH 4.3) and treated with 1.25 volumes of acetone in the cold. The resulting precipitate was separated and dissolved for assay purposes in saline solution containing phosphate buffer (final pH 7.5). Guinea pigs were injected daily for 8 days with equal doses of this preparation, sacrificed on the 9th day and examined histologically as previously described.[1] The same lesions were again observed but at dose levels usually well below those hitherto required. The results obtained with one of our most potent preparations (1H) are listed in Table I. They demonstrate a correlation between dose and intensity of biological response adequate for testing the stability of this product. Only part of its activity was destroyed at pH 7.5 upon heating on steam bath for one hour. Partial inactivation also occurred upon exposure to pH 1.1 at 27.5° for 24 hours while a solution adjusted to pH 11.2 lost most of its potency at 27.5° in 24 hours. An active solution enclosed within a Visking membrane, retained its potency upon dialysis against distilled water (66 hours at 3°). Our products contained nitrogen but only a trace of phosphorus (Preparation 1H after dialysis 12.5% N, 0.1% P). They developed an intense pink color with carbazole and sulfuric acid,[3] indicating the presence of substantial amounts of carbohydrate. Most of the nitrogen could be accounted for colorimetrically as biuret nitrogen. Treatment with trypsin (Fairchild) resulted in an increase in nitrogen not precipitable with trichloracetic acid and a loss of about ⅘ of biological activity. Although preparation 1H migrated in a single boundary in the electric field at the rate of -5.30×10^{-5} $cm^2\ volt^{-1}\ sec^{-1}$ (Tiselius apparatus; veronal buffer pH 8.53, $\mu = 0.1$, 0.5°) we cannot regard this product as homogeneous. The

TABLE I.
Biological Assay of Preparation 1H.

Urine equiv. of total dose, cc	Total No. of animals	No. of animals, with involvement of 0 organs*	1	2	3	4
50	6	5	1			
100	5		3	2		
200	3			3		
250	2			1	1	
500	9			3	6	
1000	3			1	1	1
2000	3					3

* 1 organ = spleen, 2 organs = spleen and marrow, 3 = spleen, marrow, and liver or adrenal, 4 = spleen, marrow, liver, and adrenal.

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* This work has been made possible by a grant from the Commonwealth Fund.

[1] Heinle, R. W., Wearn, J. T., Weir, D. R., and Rose, F. A., *Ann. Int. Med.*, 1942, **17**, 902.

[2] Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, 1932, **98**, 739.

[3] Gurin, S., and Hood, D. B., *J. Biol. Chem.*, 1939, **131**, 211.

spectral characteristics ($E_{420} = 0.203$, $E_{440} = 0.199$, $E_{520} = 0.286$, $E_{540} = 0.320$ per mg; cell length 1 cm) of the color produced in the carbazole reaction do not agree with those of any simple mixture of monosaccharides. Furthermore, after 0.7 saturation with sodium sulfate, the liquid phase contained significantly more activity and carbohydrate per mg of nitrogen than the precipitate.

Our data suggest that the active principle of our preparations is a protein or glycoprotein. It differs in its chemical and physical properties from the active preparation with similar or identical biological effects obtained from leukemic urines by Turner and Miller.[4] The possibility still exists that their product which is regarded as a ketoacid occurs as a prosthetic group in our preparations. However, the loss of activity which we observed on treatment with trypsin would indicate that any prosthetic group present ought to be less potent than the intact molecule.

The authors are indebted to Drs. Hicks-Bruun, Dobriner and Rhoads, Memorial Hospital, New York, for the preparation of an adsorbate of leukemic urine, to Drs. San Clemente and Ecker, Institute of Pathology, Western Reserve University, for the electrophoretic measurements, and to Dr. Gurin, University of Pennsylvania, for a preparation of carbazole.

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[4] Turner, D. L., and Miller, F. R., *J. Biol. Chem.*, 1943, **147**, 573.

14818

A Comparison of Various Methods of Demonstrating Influenza Virus in Throat Washings.*

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The detection of influenza virus in throat washings is an important method, during epidemics, of determining the causative virus as rapidly as possible. Methods of isolating influenza virus in ferrets and mice,[1] in hamsters,[2] and in chick embryos by amniotic[3] or allantoic[4] inoculation have been described, but a comparison of the efficiency of these 4 methods on the same group of throat washings has not been available. During the epidemic of influenza A in 1943-44, 83 throat washings from military personnel[5] were tested in ferrets or hamsters, or in both species, and a smaller number of the same washings were inoculated into chick embryos.

Methods. Ferrets were inoculated intranasally under light ether anesthesia with 2 cc of unfiltered throat washings. Temperatures of the animals were recorded twice daily, and blood specimens were collected from all animals before, and 10 days after, inoculation.

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* The studies and observations on which this paper is based were supported by the International Health Division of The Rockefeller Foundation, in cooperation with the California State Department of Public Health.

† Representing the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General of the United States Army.

[1] a. Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Brit. J. Exp. Path.*, 1935, **16**, 566; b. Francis, T. Jr., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1172.

[2] Taylor, R. M., and Parodi, A. S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 105.

[3] Burnet, F. M., *Brit. J. Exp. Path.*, 1940, **21**, 147.

[4] Thigpen, M., and Crowley, J., *Science*, 1943, **98**, 516.

[5] Members of the Commission on Influenza, *J. Am. Med. Assn.*, 1944, **124**, 982.

Hamsters were inoculated intranasally under ether anesthesia with 0.5 cc of unfiltered throat washings, and 2 to 3 days after the first inoculation the animals were reinoculated with the same washing according to the method described by Taylor.[2] Blood specimens were collected by cardiac puncture before the first, and 10 days after the second inoculation.

The paired serum specimens from the ferrets and hamsters were tested for an increase in antibodies by means of the Hirst chicken cell-agglutination technic,[6] using the PR8 and several recently isolated strains of influenza virus A, and the Lee strain of influenza virus B. No attempt was made to carry out passages in ferrets or hamsters. The diagnosis of influenza in most of the cases was also established by the complement fixation and chicken cell-agglutination tests on the human serums.

Initially 6 to 24 chick embryos 11 or 12 days of age were inoculated by the amniotic route with each throat washing filtered through Berkefeld N filters. With most specimens the window method of Burnet[3] was used, but with some the stab method described by Hirst[7] was also employed. Three to 5 amniotic passages at intervals of 2 to 5 days were carried out with each throat washing before the result was considered negative. Virus was detected in the amniotic or allantoic fluids by agglutination with human, guinea pig, or chicken erythrocytes[8] and was identified by inhibition tests with known type A and B antisera.

Allantoic inoculations were done with unfiltered throat washings centrifuged for 20 minutes at 4,000 R.P.M. in an angle head and also with duplicate specimens treated with Zephiran[‡][9] at a dilution of 1:20,000 for 20 to 30 minutes. Only 1 passage was done because of the overgrowth of bacterial contaminants in almost all of the embryos inoculated by this method. The virus was detected and identified by the method used for the amniotic passages. From the third to the sixth day after inoculation allantoic fluids were withdrawn daily by a needle and syringe from the living embryos and tested for agglutinins so that they might be harvested or passed if positive.

Results. The results of inoculating ferrets with throat washings from 62 patients are summarized in Table I. None of the throat washings from 23 patients with negative serological tests for influenza A produced any antibody response to that virus after intranasal inoculation into ferrets. Of the 30 throat washings from patients with positive serological tests for influenza A, 17 (56.7 ± 9.05%) produced in the ferrets an increase in antibodies to influenza virus A. With 2 exceptions all of the ferrets showing an increase in antibodies also developed a febrile reaction 2 to 3 days after inoculation. Four of the 41 ferrets showing no increase in antibodies had a similar febrile response. All ferrets were also tested for antibodies to influenza virus B, and none showed an increase.

Each of 55 throat washings was inoculated into 2 hamsters. The results are presented in Table II. Not included are 2 throat washings from serologically negative patients which were also negative in hamsters. Of the 20 throat washings that were positive in ferrets, shown in Table II, 17 (85.0 ± 7.98%) were also positive in hamsters. Thirty, or 56.6 ±

TABLE I.
Results of Inoculating Ferrets with Throat Washings.

Serological tests for influenza A	Antibody response to influenza virus A in ferrets	
	No. positive	No. negative
Positive	17	13
Not done	4	5
Negative	0	23

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[6] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[7] Hirst, G. K., *J. Immunol.*, 1942, **45**, 293.

[8] Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21**, 55.

[‡] Zephiran, manufactured by Alba Pharmaceutical Co., Inc., is a mixture of high molecular alkyl dimethyl benzyl ammonium chlorides.

[9] Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 543.

TABLE II.
Results of Inoculating Hamsters and Chick Embryos with Throat Washings.

Serology of cases	Responses of ferrets to throat washings	Antibody response in hamsters		Amniotic inoculation		Allantoic inoculation	
		No. Pos.	No. Neg.	No. Pos.	No. Neg.	No. Pos.	No. Neg.
Positive	Positive	13	3	3	8	2	7
Positive	Negative	0	12	0	3	0	1
Positive	Not done	13	8	0	2*	0	0
Not done	Positive	4	0	4	0	0	3
Total		30	23	7	13	2	11

* These 2 throat washings were positive in hamsters.

6.81%, of the 53 specimens from known cases of influenza A were positive in hamsters, a result which is identical with that obtained in ferrets. Twelve throat washings that were negative in ferrets produced no antibody response in hamsters.

For the purpose of determining the effects of antigenic variations the pre- and post-inoculation serums from all of the ferrets, and a large proportion of the hamsters, receiving throat washings were tested with 2 or more strains of influenza virus A isolated during the epidemic of 1943-44 in addition to the strain PR8. Two ferrets failed to develop a definite antibody response to the strain PR8, but did show an increase in antibodies to the recently isolated strains. This was also true of 2 sets of hamsters inoculated with other throat washings. In one or two instances the antibody response of the animals was negative with one of the recently isolated strains, but positive with others and with PR8.

The results of inoculating chick embryos are summarized in Table II. Seventeen, or all but 3, of the throat washings inoculated by the amniotic route, had been shown to contain virus by tests in ferrets or hamsters. Of these 17 specimens, 7 (41.2 ± 11.9%) infected chick embryos amniotically. Virus was demonstrated in 4 throat washings in the first passage and in 3 on the second passage, but usually 1 or 2 additional amniotic or allantoic passages were necessary before the virus reached sufficient titer to be positively identified as influenza A. In the early amniotic passages of 2 strains the titer of amniotic fluid was higher with guinea pig cells than with chicken cells. This is in accord with the observations of Burnet.[8]

Virus was demonstrated in 2 out of 13 unfiltered throat washings on the first allantoic passage. Ten of the same 13 throat washings were treated with Zephiran 1:20,000 before inoculation into the allantois of chick embryos. In one case virus was isolated from both Zephiran treated and untreated throat washings. The strain of virus isolated from the Zephiran treated throat washings was carried for 4 allantoic passages with the addition of Zephiran. A streptococcus contaminated each of the passages and it was necessary to filter the fourth passage material before carrying the virus further in chick embryos. A similar procedure was followed with the other strain isolated from an untreated throat washing.

Discussion. The results indicate that hamsters and ferrets are probably equally reliable as experimental animals for the demonstration of influenza A virus in human throat washings, as judged by the antibody response of these animals. Chick embryos may have been less reliable, but they afforded the advantage of more rapid isolation of virus.

Demonstration of virus in ferrets and hamsters required 10 to 14 days, a period of time equal to that required for serological diagnosis with the human serum. Isolation of virus from the first passage ferrets could be accomplished by passing nasal washings to mice or chick embryos according to the method of Burnet,[8] and from hamsters by passage of lungs and turbinates to mice according to the method of Taylor and Parodi.[2]

With chick embryos the required time for identification of the virus was 3 to 10 days, depending on the number of passages before positive agglutinations of red cells were obtained. With about 5 to 10% of the throat washings it was possible to identify the virus within 3 to 4 days, using either the amniotic or allantoic inoculation. Zephiran at a concentration which would not inactivate the virus was not consistently effective in removing bacteria. It is quite possible that different results in ferrets, hamsters, and chick embryos would be obtained with throat washings from patients with influenza B.

Summary. During the epidemic of influenza A in 1943-44, throat washings from military personnel were tested in ferrets or hamsters, or in both species, and a smaller number of the same washings were inoculated into chick embryos.

None of the throat washings from 23 persons in whom response to serological tests for influenza A virus was negative produced antibodies to that virus after intranasal inoculation in ferrets.

Of 30 throat washings from persons showing positive response to serological tests for influenza A virus, 17 (56.7 ± 9.05%) produced in ferrets an increase in antibodies to the virus. With 2 exceptions all ferrets showing an increase in antibodies also developed fever 2 to 3 days after inoculation. Four of 41 ferrets showing no increase in antibodies had similar febrile reaction.

Thirty, or 56.6 ± 6.81% of 53 throat washings from persons with known cases of influenza A were positive in hamsters, a result identical with that obtained in ferrets. Twelve throat washings that were negative in ferrets produced no antibody response in hamsters.

Seventeen throat washings shown to contain influenza A virus by tests in ferrets or hamsters were inoculated into chick embryos by the amniotic route. Of these, 7 (41.2 ± 11.9%) infected the embryos. Virus was demonstrated in 4 throat washings in the first passage and in 3 in the second, but usually 1 or 2 additional amniotic or allantoic passages were necessary before the virus reached sufficient titer to be positively identified as influenza A.

In 2 of 13 unfiltered throat washings inoculated into the allantois of chick embryos, virus was demonstrated on first passage.

14819 P

Electrocardiographic Changes in Epidemic Parotitis (Mumps).*

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In January, 1943, a 20-year-old white sailor entered the hospital with epidemic parotitis and on the fifteenth day of his illness complained of constant retrosternal pain and of dizziness on arising. His pulse rate was 35 per minute and the electrocardiogram revealed complete heart block with prolongation of the QRS interval (0.12 sec.). He improved rapidly and by the eighth day following the onset of his angina the electrocardiogram was normal. Early this year, another 20-year-old white sailor, under treatment for mumps, developed precordial pain and palpitation on the seventh day of illness. The pulse rate fell to 28 per minute and the electrocardiogram showed 3:1 heart block. Three days later, another electrocardiogram revealed complete auriculo-ventricular dissociation. His condition slowly improved and by the 77th day following the onset of his cardiac pain the electrocardiogram had returned to normal. In both instances the leucocyte counts and sedimentation rates were elevated during the acute phase. A review of the literature revealed no reference to any similar observations. Hence, a study of the electrocardiographic findings in mumps was undertaken, in order to determine the incidence, variations and prognostic significance of the electrocardiographic changes.

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* This article has been released for publication by the Division of Publications of the Bureau of Medicine and Surgery of the U. S. Navy. The opinions and views set forth are those of the writer and are not to be considered as reflecting the policies of the Navy Department.

Method. Electrocardiographic tracings were taken on a series of 104 adults with mumps during the height of an epidemic early in 1944. Standard limb leads and chest lead CF4 were used in all cases. In 46 of these patients serial electrocardiograms were made at intervals of 2 to 4 days. This study was correlated with determinations of the sedimentation rate and with blood counts. In addition, a careful search was made for clinical evidence of cardiac involvement.

Results. Significant electrocardiographic findings were observed in 16 of the 104 patients (15.4%). In 4 of these, the P waves became diphasic or inverted in one or more leads. In 2 individuals, prolongation of the P-R interval was noted. In 1 instance, the QRS complex in CF4 became inverted, and in another, the voltage in the limb leads decreased. Two patients showed considerable elevation of the S-T segments in CF4, and in 1 of these the S-T segment was bowed upwards. In 1 subject the S-T segment became depressed beyond normal in leads II and III. Changes in the T waves were observed in all of the 16 patients; in 11 of these they were noted in two or more leads. The T waves became tiny, diphasic or inverted and with serial electrocardiograms the evolutionary stages in the development of T wave inversion were demonstrable. T wave abnormalities occurred with essentially the same frequency in all 4 leads. In 14 out of the 16 patients with electrocardiographic changes more than one abnormality was found; in the remaining 2, the T wave changes were decisive. No specific electrocardiographic pattern was recognizable in this study.

In all but one patient the electrocardiographic abnormalities were observed on the fifth to the tenth day of illness, and in the majority of these were present on the eighth or ninth day. In 1 subject an abnormal tracing was found on the twentieth day. The changes were transitory in 14 patients and returned to normal in 2 to 35 days. However, in most instances the curves were normal in 4 to 8 days. In the remaining 2 patients electrocardiographic evidence of progressive improvement was observed, but residual changes persisted for 3 months and 5 months respectively.

Precordial pain, either alone or with dyspnoea and palpitation, developed in 4 patients and in 2 of these antedated the appearance of the electrocardiographic abnormalities. A soft blowing systolic murmur became audible at the mitral area in 2 patients but disappeared in 2 months. The sedimentation rate was elevated in 5 individuals and a slight leucocytosis was found in 4.

Comment. Cardiac involvement in epidemic parotitis has generally been regarded as a rare complication and, when observed, has been described as an endocarditis or pericarditis. That mumps may also produce myocarditis was originally suspected by Pujol[1] and by Barbato[2] from clinical observations, but no proof of their contention was presented. Later, Manca[3] described an instance of acute interstitial fibrinous myocarditis which he regarded as a characteristic reaction to the mumps virus. Since the completion of this work, Wendkos and Noll[4] reported a single instance of mumps with T wave changes and prolongation of the P-R interval.

It is evident from the present study that myocardial involvement in mumps is not a rare event and that the electrocardiographic changes are similar to those observed in rheumatic fever. Moreover, the occurrence of delayed A-V conduction time in epidemic parotitis casts further doubt on the specificity of this finding and raises the question of its reliability in the diagnosis of rheumatic fever. Rather, it is added confirmation of the belief that the myocardium is susceptible to involvement not only in rheumatic fever and mumps but in other forms of generalized bacterial or virus infections as well. These implications and other aspects of the problem will be dealt with in greater detail in the final report.

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[1] Pujol, M., *Arch. de med. et pharm. mil.*, Paris, 1918, **69**, 527.

[2] Barbato, M., *Rif. Med.*, 1925, **41**, 1109.

[3] Manca, C., *Arch. ital. di anat. istol. pat.*, 1932, **3**, 707.

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Summary. 1. Two isolated cases of epidemic parotitis accompanied by electrocardiographic evidence of complete heart block are reported. 2. In a subsequent study of 104 consecutive cases of epidemic parotitis in adults, evidence of myocardial involvement was observed in 16 instances (15.4%). In all but 2 patients, the electrocardiographic changes were transitory and of short duration. The broad implications of these findings are discussed briefly.

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Inhibition of Drug Precipitation in the Urinary Tract by the Use of Sulfonamide Mixtures. I. Sulfathiazole-Sulfadiazine Mixture.*

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Serious toxic effects of sulfonamides upon the kidneys have appeared only after introduction of N^1-heterocyclic derivatives of sulfanilamide. Preference of these compounds over sulfanilamide in human therapy is based primarily on their wider range of antibacterial activity. Two representatives of this group, sulfathiazole and sulfadiazine, are employed at present as the "drugs of choice" whenever sulfonamide therapy seems indicated, despite their poor solubility if compared with the mother substance sulfanilamide. Hence renal concrement formation—a complication unknown from sulfanilamide—has become a serious problem worthy of the most careful attention.

The present investigation deals with the possibility of lessening the danger of renal concrement formation by the employment of sulfonamide mixtures. Solubility studies with N^1-heterocyclic derivatives of sulfanilamide revealed that these compounds are soluble in one and the same solution nearly to the extent of their separate solubilities; in other words, a saturated solution of sulfadiazine, for instance, can still be further almost fully saturated with sulfathiazole. This finding applies even to compounds which are structurally as closely related as sulfadiazine and its monomethyl and dimethyl derivatives (sulfamerazine and sulfamethazine). Table I illustrates these observations. Almost identical results were obtained in solubility studies with normal human urine.

Since even closely related sulfonamides, when present simultaneously in the same solution, do not influence each other appreciably with regard to their particular solubilities, the danger of intrarenal drug precipitation from the sulfonamides comprising the mixture should be only as great as if each compound had been administered alone, and in the partial dosage contained in the mixture. On the other hand, it seemed probable that in using combinations of different sulfonamides having similar or identical therapeutic indications, the antibacterial effect would largely correspond to the total concentration of free sulfonamide.

The validity of these contentions was investigated in toxicity experiments with albino rats and with the help of *in vitro* antibacterial studies. Sulfathiazole and sulfadiazine were selected for the initial series of experiments, because these compounds are at present most frequently employed, have almost the same therapeutic indications, and cause renal complications in about the same percentage of

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* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

TABLE I.
Solubility of Sulfonamide Mixtures.*
Sulfathiazole (ST), sulfadiazine (SD), sulfamerazine (SMD), and sulfamethazine (SMMD), and mixtures of these compounds in powdered form were shaken in water for one hour at room temperature (20°C). The fluids, which always contained a safe excess of the drugs, were then filtered and the sulfonamide concentration in the filtrate was determined in a 1:200 dilution according to the method of Bratton and Marshall using a Klett Summerson Photo Colorimeter.

		Sulfonamide dissolved, mg %	
Drug	Reading	Found	Expected (Sum of solubilities of the single drugs)
SD	36	7.3	
SMD	89	20.2	
SMMD	163	43.0	
ST	159	34.4	
SD + SMD	120	25.6	27.5
SD + SMMD	200	50.3	50.3
SD + ST	180	38.3	41.7
SMD + SMMD	232	58.1	63.2
SMD + ST	230	50.7	54.6
SMMD + ST	300	72.0	77.4
SD + SMD + SMMD + ST	395	93.0	104.9

* Grateful acknowledgment for liberal supplies of the drugs used is made to the Schering Corporation, Bloomfield, N.J. (ST); the Lederle Laboratories, New York, N.Y. (SD and SMD); and to Sharp & Dohme, Philadelphia, Pa. (SMD and SMMD).

cases in experimental animals as well as in humans.[1-6]

Results. From previous experience it was known that both sulfathiazole and sulfadiazine are readily precipitated out in the urinary tract of albino rats if given in large amounts, and that renal obstruction from drug precipitate plays an important part in causing death from sulfathiazole or sulfadiazine intoxication.[7-9] It was expected, therefore, that joint administration of the two compounds, in amounts comparable in total weight to fatal dosages of either drug when administered separately, would reveal a significantly lower toxicity because of a diminished tendency to intrarenal precipitation.

Experimental results confirmed this viewpoint. It was found that the acute and chronic toxicity of sulfathiazole-sulfadiazine mixtures is strikingly low if compared with the toxicity of each of the two compounds when administered separately. The findings are briefly summarized in Table II.

Evidence derived from chemical studies pointed to a significant decrease of renal obstruction, following joint administration of the 2 sulfonamides, as the chief reason for the strikingly low toxicity of the mixture. This could be inferred in particular from a comparison of the nonprotein nitrogen levels in the blood and from comparative follow-up studies of the sulfonamide concentration in blood and urine at predetermined intervals. The sulfonamide blood levels obtained with the mixture were, as expected, much higher than from sulfathiazole and only slightly lower than from sulfadiazine. The average total sulfonamide values (mean of 10 animals) for the dosages used in the acute toxicity study (Table II) were, after 24 hours: for sulfadiazine 121 mg %, for sulfathiazole 45 mg %, and for sulfadiazine-sulfathiazole 85 mg %.

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[2] Peterson, O. L., and Finland, M., *Am. J. M. Sc.*, 1941, **202**, 757.

[3] Simon, M. A., *Am. J. M. Sc.*, 1943, **205**, 439.

[4] van Dyke, H. B., *Ann. New York Acad. Sc.*, 1943, **44**, 477.

[5] Callomon, F. T., *J. Lab. and Clin. Med.*, 1944, **29**, 574.

[6] Vilter, C. F., and Blankenhorn, M. A., *J. A. M. A.*, 1944, **126**, 691.

[7] Lehr, D., Antopol, W., and Churg, J., *Science*, 1940, **92**, 434.

[8] Lehr, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 82.

[9] Lehr, D., *Bull. New York Acad. Med.*, 1944, **20**, 424.

TABLE II.
Comparative Toxicity in Albino Rats of the Sodium Salts of Sulfadiazine (NaSD), Sulfathiazole (NaST), and Mixtures of the Two Compounds.

Acute Toxicity.

Drug, g per kg body wt				
Single intraperitoneal inj. of	Sulfonamide total amt	No. of animals	% death	Remarks
NaSD, 1.5	1.5	10	90	Died within 1 to 3 days
NaST, 1.1	1.1	15	67	" " 1 " 4 "
NaSD, 0.75 NaST, 0.55	1.3	10	0	Mixture of ½ of each of above dosages. Complete recovery.

Chronic Toxicity.

Drug, g per kg body wt				
*Daily intraperitoneal inj. of	Sulfonamide total amt daily	No. of animals	% death	Remarks
NaSD, 0.9	0.9	10	100	Died within 3 to 6 days
NaST, 0.9	0.9	10	100	" " 1 " 4 "
NaSD, 0.45 NaST, 0.45	0.9	10	40	" " 3 " 7 "

* The animals were injected for a maximum of 6 consecutive days.

After the same interval, the urinary drug excretion per rat (mean of 10 animals) had reached 29 mg for sulfadiazine, 34 mg for sulfathiazole and *72 mg* for sulfadiazine-sulfathiazole. The strikingly high renal excretion of the mixture from blood levels distinctly lower than in the sulfadiazine group, suggested a diminution or even absence of renal obstruction in the animals receiving the sulfonamide combination. Further support for this viewpoint was derived from the marked difference in the nonprotein nitrogen values of the surviving rats 48 hours after the drug injection (mean values: sulfadiazine group 182 mg %, sulfathiazole group 120 mg %, sulfadiazine-sulfathiazole group *82 mg %*), and also from post-mortem examinations, especially of the urinary tract, which disclosed that the mixture animals showed little or no renal damage.

The *in vitro* antibacterial efficacy of solutions containing equal parts of sulfathiazole and sulfadiazine, as compared to those holding the same total concentrations of either drug alone, was tested thus far against strains of *Streptococcus hemolyticus*, *Staphylococcus aureus* and pneumococcus. Against the streptococcus the sulfadiazine-sulfathiazole combination was significantly more effective than either of the 2 drugs alone, whereas against the staphylococcus and pneumococcus, the mixture was more active than sulfadiazine but less active than sulfathiazole.

The results from the animal experiments and the *in vitro* studies suggest that the employment of combinations of sulfathiazole and sulfadiazine in human therapy (for instance, half the therapeutic dose of each), might considerably lessen the danger of concrement formation in the urinary tract, presumably to the extent as if only half the therapeutic dose of the one *or* the other of the 2 compounds had been administered. The *in vivo* bacteriostatic activity of the combination, however, will probably match the effectiveness of either sulfathiazole or sulfadiazine and might in some instances even surpass it. The clinical evaluation is under way. The merit of combining sulfadiazine and sulfathiazole in the treatment of pneumonia, for reasons of rapidly obtaining and maintaining satisfactory sulfonamide levels in the blood, has been demonstrated recently.[10]

The findings of the solubility study summarized in Table I indicate that many other sulfonamide combinations should be investigated as to their ratio of renal toxicity and bacteriostatic activity.

Summary. It was observed that N^1-heterocyclic derivatives of sulfanilamide, even if closely related, are soluble in one and the same solution almost to the extent of their separate solubilities. Based on this observation, the toxicity and antibacterial activity of sulfadiazine-sulfathiazole mixtures was investigated. It was found that the acute and chronic toxicity of this drug combination for albino rats is strikingly low if compared with the effect of similar or identical total concentrations of either sulfathiazole or sulfadiazine alone. Evidence derived from chemical analyses and post-mortem examinations pointed to a diminution of intrarenal obstruction from drug precipitate as the chief reason for the low toxicity of the mixture. *In vitro* antibacterial studies showed that the effectiveness of the mixture corresponded largely to the total concentration of free sulfonamide. On the basis of these observations, the substitution at the bedside of sulfathiazole or sulfadiazine by combinations of these two compounds was suggested, because their application might decrease the danger of renal complications without interfering with the antibacterial efficacy. Clinical trials are in progress.

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The author wishes to express his appreciation to Miss Helen Salzberg, Miss Catherine Russel, and Miss Ruth Levi for valuable technical assistance.

14821

Poliomyelitis Virus in the Blood Stream in the Experimental Disease.*

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Although most attempts to demonstrate poliomyelitis virus in the blood of experimentally-infected monkeys have been unsuccessful, four positive tests have been reported,[1,2,3,4] 3 of which were described more than 30 years ago. In all 4 instances the blood was drawn soon after the onset of paralysis. Furthermore the virus has long been sought in human blood but with the exception of a single instance, in spite of many trials,[5] such tests have been negative.[6]

The present data are placed on record to indicate some of the conditions under which poliomyelitis virus has been detected in the blood of monkeys during a recent series of experiments. In addition the virus has been looked for in the colon contents of many of these monkeys and the results of these tests are also presented.

Methods. Rhesus (*Macaca mulatta*) and capuchin (*Cebus capucina*) monkeys were used. The monkeys used as donors had been infected by the intracerebral, intracutaneous, and other routes. Three strains were used—all in their early passages. These strains originated in human stools of poliomyelitic patients from Texas (1942), Hartford (1942), and various sections of the country (1940-1941). The history of these strains in their early passages through monkeys has been described.[7]

Blood was collected from the heart and no attempt was made to prevent clotting. The whole blood was either stored on dry ice or it

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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TABLE I.
Poliomyelitis Virus in Monkey Blood and Colon Contents.

Inoculum			Donor monkey No.‡	Result of inoculation		Day blood and colon contents collected	Results of virus test in		
Strain-Generation*	Material	Route†		Onset of fever§	Onset of paralysis‖		Blood¶	Colon contents¶	Spinal cord¶
Hartford i	Stool	IC	R-2259	7	10	11	+	—	
" i + ii	Spinal cord	IQ	R-2300	0	8	9	+	+	+
" iii + iv	" "	IC, IP IN, IQ SQ	C-2324	9	11	11	+	—	+
" ii + iii	Stool	IC	R-2370	10	11	12	+		
" ii	Blood	IC	R-2274	11	13	15	—		
" i + ii	Spinal cord	IQ	R-2299	12	13	13	—	—	
" ii + iii	Stool	IC	R-2335	0	19	19	—	—	
U.S. i	Spinal cord	IC	R-2338	14	16	16	—		
Texas i	" "	IC, IP IQ, SQ	C-2356	7	10	11	—	+	+
" o	Stool	IC	R-2289	7	10	11	—	—	+

* o = original human material was inoculated; i = material from monkey representing the first generation of the virus in monkeys was inoculated, etc.

† IC = intracerebral; IQ = intracutaneous; IN = intranasal; SQ = subcutaneous; IP = intraperitoneal.

‡ R = rhesus (*Macaca mulatta*) monkey; C = capuchin (*Cebus capucina*) monkey.

§ 7 = fever occurred 7 days after inoculation; 0 = no fever observed.

‖ 10 = paralysis first observed 10 days after inoculation.

¶ + = positive test for poliomyelitis virus; — = negative test for poliomyelitis virus. One monkey was used for each test.

was immediately prepared for inoculation. To the volume of blood drawn—30 to 90 cc—enough water was added to make a total volume of 120 cc; the hemolyzed blood was mixed in a Waring blendor for about 5 minutes. It was then centrifuged at 3000 RPM for 20 minutes. 90 cc of the supernatant fluid was subjected to ultracentrifugation at 39,000 RPM for 60 minutes. The gelatinous pellets were taken up in 2 to 3 cc of water, spun lightly in the laboratory centrifuge, and 1 to 2 cc inoculated intracerebrally into a rhesus monkey. Colon contents, taken at autopsy immediately after the blood was drawn, were also prepared by ultracentrifugation as outlined in a previous paper.[8] These samples were also inoculated intracerebrally, one monkey being used for each test. Spinal cord was passed as a 10% suspension and was inoculated intracerebrally and intraperitoneally.

The criterion for a positive test (*i.e.*, demonstration of virus in blood, colon contents, or spinal cord) was the production in the test animals of typical histological lesions, viz., neuronophagia and neuronal necrosis associated with perivascular and interstitial cellular infiltration.

Results. In this series of experiments (Table I) virus was found in the blood stream of 4 out of 7 monkeys infected with the Hartford strain and in none out of 3 monkeys infected with other strains. Two of the monkeys with viremia had been inoculated *intracerebrally* with the stools of experimentally infected monkeys whereas the other 2 had been inoculated with infected spinal cord. Of the latter 2 monkeys, one, a rhesus, had received an *intracutaneous* inoculation and the other, a capuchin, had received multiple inoculations by intracerebral as well as by other routes. The colon contents of 3 of these 4 monkeys were also tested for virus and only one yielded a positive result. The spinal cords of 2 of these monkeys were passed successfully.

Tests were carried out on the colon contents of 4 of the monkeys yielding negative blood tests; one was positive.

The specimens recorded as positive in Table

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[8] Melnick, J. L., *J. Exp. Med.*, 1943, **77**, 195.

I produced paralysis in the test monkeys in all cases but one. This latter monkey had the non-paralytic form of the disease as evidenced by a negative clinical course coupled with poliomyelitic lesions in its spinal cord.

Comment. The attempt will not be made to determine the significance of finding virus in the blood of 4 of 10 paralyzed monkeys. There is no reason to suspect that this represents an extra-neural pathway for the primary distribution of virus in the body; it may well be the direct result of the overflow or of the breakdown of virus-laden nerve cells. The distribution of lesions[9] and of virus[10] only in certain regions of the central nervous system both in the experimental and human disease would lead one to favor the view that virus in the blood does not play a predominant role in determining the location of virus within the central nervous system, once the latter has gained access to this tissue. In these experiments the presence of *detectable* amounts of virus in the blood stream was neither a prerequisite nor a predisposing factor for the appearance of *detectable* amounts of virus in the colon contents during the acute disease.

All of the positive results were found in monkeys inoculated with one strain (Hartford) of poliomyelitis virus. Three tests on two other strains were negative. This finding may recall the fact that certain strains, usually in their early passages, are more infectious by the cutaneous route than are others.[11,12]

Summary. Of 10 monkeys, paralyzed after being inoculated with poliomyelitis virus of recent human origin, three rhesus and one capuchin monkey were found to have the virus in the blood stream. All of the positive tests in the blood were due to a single strain. The colon contents of three of these latter monkeys were examined for virus and it was found in only one instance. Of 4 monkeys in whose blood virus could not be detected, one was found to have virus in its colon contents.

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14822

Effect of Aspergillic Acid on Equine Encephalomyelitis Virus.*

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During the current outbreak of equine encephalomyelitis among horses in the Southwest, a few human cases occurred.[1] This prompted study on treatment of the disease, since no effective therapeutic agent is available. Although antiserum may be effective in the treatment of horses when administered during the early stages of the disease, such therapy is of little value in man because the central nervous system lesions are usually too far advanced at the time when the diagnosis of encephalitis is made. A study of the effect of aspergillic acid on a neurotropic virus seemed of interest because, to our knowledge, no reports have appeared in the literature concerning the effect of this substance on viruses, despite its efficacy on a wide variety of bacteria.[2] Also, there are indications that this drug diffuses rapidly into the central

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* This work was aided by a grant from Eli Lilly and Company, Indianapolis, Indiana.

[1] Sulkin, S. E., and Ashworth, C. T., to be published.

[2] Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1944, **45**, 461.

TABLE I.

In vitro Effect of Aspergillic Acid on Equine Encephalomyelitis Virus (Western Type).

Experiment No.	Procedure*	Dil. of virus suspension	Results§
1	Virus and aspergillic acid†	10^{-7}	2,2,2,2
	” alone (control)		2,2,2,2
2	” and aspergillic acid	10^{-8}	3,S,S,S
	” alone (control)		2,2,3,S
3	” and aspergillic acid	$10^{-8.5}$‡	3,3,3,4,5,S,S,S,S,S
	” alone (control)		2,3,3,3,3,5,S,S,S,S

* Virus and drug shaken at room temperature for 3 hours; mixture injected intracerebrally into 3-week-old mice (inoculum 0.03 ml).

† 1 mg aspergillic acid per ml virus suspension.

‡ Intracerebral LD_{50} titer.

§ Each figure represents one mouse and the day of death. S = mouse survived.

nervous system when injected intraperitoneally into mice.[3]

The following experiments are concerned primarily with the *in vitro* effect of aspergillic acid* on the virus of equine encephalomyelitis. The Western strain of equine encephalomyelitis virus obtained from Dr. W. McD. Hammon in 1943 was used. The virus has been maintained by intracerebral injection in mice. Before the experiments were started several passages were made in mice to insure a high virulence. The cerebral titer was determined by the 50% end point of death of 3- to 4-week-old mice.

In the first experiment a 10^{-7} dilution of infected mouse brain from a moribund animal was prepared, and aspergillic acid was added as the sodium salt in the arbitrary amount of 1 mg per ml of virus suspension. The virus and drug mixture, together with virus suspension alone, which served as control, were shaken at room temperature for 3 hours. The virus and drug mixture, together with virus suspension, was then injected intracerebrally into 3-week-old Swiss mice (inoculum 0.03 ml). A titration of the virus suspension was carried out simultaneously; the intracerebral LD_{50} was found to be approximately $10^{-8.5}$/0.03 ml. The aspergillic acid appeared to have no effect whatever on the virus suspension used, since all animals inoculated with the virus-drug mixture died within 2 days, as did the controls receiving virus alone (Table I). All animals showed typical symptoms of encephalitis. Since the concentration of virus used in this *in vitro* experiment seemed too severe an inoculum, the number of lethal doses was reduced. In the second experiment, the drug was added to a 10^{-8} dilution of mouse brain and the procedure was repeated as above. Three out of 4 animals receiving virus-drug mixture survived the observation period of 15 days, while only one of the animals receiving virus alone survived. These results prompted us to undertake an experiment using a larger number of animals. In this experiment (No. 3) the drug was added to a $10^{-8.5}$ dilution of virus (LD_{50}), and the procedure was repeated as above. The results of this experiment indicate that the differences obtained in experiment 2 were probably due to chance.

In conclusion, then, these experiments indicate that a contact for 3 hours at room temperature with aspergillic acid had no effect on the infectivity of the equine encephalomyelitis virus.

Although it seemed improbable that favorable therapeutic results could be expected from the use of aspergillic acid, one such experiment was conducted. Twenty mice were infected by intracerebral inoculation of 0.03 ml of 10^{-7} suspension of Western type of equine encephalomyelitis virus. This dose of virus was used because in man the virus is usually present in high concentration before the diagnosis is made and therapy instituted. Ten mice received aspergillic acid in the amount of 50 mg per kilogram intraperitoneally immediately following the inoculation of the virus and twice daily thereafter. All mice died within 3 days, the treatment thus being completely ineffective.

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[3] White, E. C., and Hill, J. H., *J. Bact.*, 1944, **45**, 433.

* Obtained from the Squibb Institute for Medical Research, New Brunswick, N. J., through the courtesy of Miss Clara M. McKee.

Conclusion. Aspergillic acid in high concentration had no effect *in vitro* on the infectivity of the Western type of equine encephalomyelitis virus. There was also no effect on the course of the infection in mice.

14823 P

Relation of the Milk Influence to the Carcinogenic Induction of Mammary Cancer in Mice.*

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The administration of carcinogens to mice of inbred strains may result in the appearance of epitheliomas, sarcomas or other tumors to which the animals show no spontaneous susceptibility. In certain strains in which lung tumors, hepatomas, leukemia, or mammary cancer appear spontaneously carcinogens have accelerated their onset.[1,2,3,4] This suggests that the synthetic carcinogen might, in these instances, potentiate the spontaneous mechanism of carcinogenesis.

Three known influences are of etiologic significance in the development of mammary cancer of the "genetic" type which occurs in high incidence in certain strains of mice.[5] These factors are (1) a genetic susceptibility, (2) an hormonal influence, and (3) a milk influence, which is a substance contained in the breast milk of high mammary cancer strains.

The relationship of the carcinogen-induced to the spontaneous neoplasm can be studied in the case of mammary cancer by using the dba strain, in which mammary cancer appears spontaneously and can also be induced by the administration of one of the carcinogenic hydrocarbons, methylcholanthrene.[6] The present experiments were designed to test the role of the milk influence in governing the response of the mammary tissue to carcinogens. Previous experiments with these mice demonstrated that the mammary gland stimulation coincidental with forced breeding was an important factor in conditioning the neoplastic response to methylcholanthrene.[7]

Strain dba males† were crossed with C3H females possessing the milk influence (Z stock) and with C3H females lacking the milk influence (ZB stock). The F_1 hybrid animals so obtained were observed as either virgin or as forced-bred females during treatment with methylcholanthrene. Breeding females of the genetic constitution (ZB♀ × dba♂)♀ × dba♂ were also skin-painted with methylcholanthrene. Of the factors necessary for the development of spontaneous mammary cancer,

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* This investigation has been aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research and the Cancer Fund of the Graduate School of the University of Minnesota.

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[6] Engelbreth-Holm, J., and Lefèvre, H., *Cancer Research*, 1941, **1**, 102.

† Sublines 12 and 212.

[7] Kirschbaum, A., Lawrason, F. D., Kaplan, H. S., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 141.

TABLE I.

Table Summarizing Data on Induction of Mammary Cancer in Mice With and Without Milk-Influence.

Genetic type	Milk-influence				No milk-influence			
	No.	Negative	Mammary cancer	Nodules	No.	Negative	Mammary cancer	Nodules
Breeding ♀'s.								
C3H ♀ × dba ♂	6	0	5*	1*	16	10	1†	5†
dba	27	0	27	—				
(C3H ♀ × dba ♂) ♀ × dba ♂					15	5	5†	5†
Virgin ♀'s.								
C3H ♀ × dba ♂	7	6	1	0	28	28	0	0
dba	31	27	4	0				
C3H	12	0	0	0				

* Latent period of carcinogenic induction 110-145 days for mammary cancer; 0.25% solution of methylcholanthrene in benzene used. Tumors were large and grew to the same size as spontaneous tumors in dba mice. Carcinogen was applied to the skin two to three times weekly.

† Latent period of carcinogenesis always in excess of 200 days. All tumors except one were smaller than those seen in test animals possessing the milk influence.

these backcross animals lacked only the milk-influence. Virgin females of the C3H stock, which develops mammary cancer spontaneously as virgins, were similarly treated with methylcholanthrene.

The results are recorded in Table I. "Nodules" were early cancerous lesions histologically, and were always multiple. Any lesion less than 3 mm in diameter was termed a "nodule" rather than a cancer. In contrast to the more or less uniform histologic appearance of adenocarcinoma in the dba strain, the induced mammary cancers exhibited a variety of histologic patterns. Squamous metaplasia within both "nodules" and larger carcinomas was characteristic of carcinogen-induced mammary cancer. In two instances the stromal tissue of a carcinoma showed sarcomatous change.

Mammary cancer or cancerous nodules were induced in the absence of the milk-influence. Carcinogenic induction of mammary cancer in low cancer strains has been reported,[8,9,10] but in these experiments the milk-influence was not a controlled factor.

In genetically identical mice (212-dba) the histologic pattern of induced mammary cancer differed in certain respects from that of the spontaneous neoplasm, suggesting a difference in the spontaneous and induced diseases. However, if breeding females treated with methylcholanthrene possessed the milk-influence, they developed mammary cancer earlier, in greater frequency, and with more vigorous growth of the cancer than in genetically identical animals lacking this influence. This would suggest a relationship between the spontaneous and induced diseases.

Preliminary data suggest that susceptibility to the carcinogenic induction of mammary cancer may be strain specific; positive correlation with susceptibility to spontaneous mammary cancer may or may not exist. Preliminary observations indicate that breeding females of one high mammary cancer strain exhibited a precocious appearance of mammary cancer under the influence of methylcholanthrene, whereas breeding females of one other high cancer strain proved resistant to the accelerated onset of the same neoplasm.

The incidence of carcinogen-induced mammary cancer in strain dba females and in hybrid females, resulting from crossing strains dba and C3H, was greatly reduced if the test animals were not permitted to breed.

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[8] Kirschbaum, A., and Strong, L. C., *Cancer Research*, 1942, **2**, 841.

[9] Strong, L. C., and Williams, W. L., *Cancer Research*, 1941, **1**, 886.

[10] Orr, J. W., *J. Path. and Bact.*, 1943, **55**, 483.

14824 P

Alterations in C.N.S. Associated with Chronic Motor Disabilities Induced by O_2 at High Pressure.

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The recovery of animals from the effects of exposure to O_2 at high pressure (OHP) is frequently remarkably rapid but residual effects commonly persist for some time after the animal's return to atmospheric pressure, varying from periods as short as a few minutes up to several days; and in some cases disabilities become permanent.[1] The post-decompressional manifestations of O_2 poisoning may then be appropriately designated as acute and chronic after-effects.

The acute after-effects are evident during the stages of decompression and the immediate post-decompressional period. These vary widely in type and intensity and in large part, may simply represent a continuation of those reactions precipitated by the OHP before decompression was begun.

The chronic effects, characterized chiefly by persistent motor disabilities and various degrees of paralyses,[1] must represent a continuation of at least some of the features of the acute after-effects. The nature of these chronic motor disabilities is highly suggestive of permanent damage to the nervous system. Yet heretofore no significant pathological changes have been demonstrated in the C.N.S. of animals which have suffered poisoning by OHP and it has been inferred that the effects of OHP on the C.N.S. are entirely "functional" in character.[2]

The failure to demonstrate lesions in the C.N.S. in poisoning by OHP[3] may perhaps be best explained by the fact that studies have been confined to the acute stages and that the induction of chronic disabilities by a single exposure is an infrequent occurrence. The fact, however, that such chronic changes can be regularly induced in experimental animals by successive exposures to OHP, should provide particularly good opportunity for demonstrating any changes which OHP might possibly induce in the C.N.S.

An investigation was therefore begun to determine what alterations, if any, might be found in animals in which chronic motor disabilities were induced by successive exposures to O_2 at pressures of about 65 pounds (gauge). The exposures, for the most part, were made 3 times per day, but neither the duration of each nor the total number employed, was uniform for all animals. This lack of uniformity in all experiments was occasioned by the individual variations in the susceptibility and by the desire to induce various degrees of severity of chronic involvement. The greater number of the exposures, however, were of about 20 to 30 minutes duration and the total number of exposures in any one series varied from as few as 4 up to 30.

Decompression was carried out in stages and occupied a period of time equivalent to that of the duration of the exposure to the increased O_2 pressure. This relatively slow decompression eliminated possible complications from O_2 bubble formation, although according to some authors such precautions are quite unnecessary. When the desired severity of chronic neuromuscular disability had been induced and had persisted for some time the animals were killed by cardiac puncture and the C.N.S. removed for histological study.

Microscopic examination of the C.N.S. tissues revealed the following significant points: congestion, perivascular oedema, oedema of the white matter, and focal areas of anemic necrosis. Neuropathological examination in other animals revealed definite pathology characterized by a softening of the white matter; and degenerative changes. The C.N.S. of those animals which succumbed shortly after a single, or the first few exposures, showed no significant alterations from that of the controls; the same was true of those which, while they were subjected to more exposures, were killed shortly after the occurrence of motor disability. It would appear therefore that animals must survive the C.N.S. injury induced by successive exposure to OHP for some time in order to permit the development of demonstrable cytological changes. This supports the explanation, mentioned above, for the failure of earlier investigations to demonstrate parenchymal damage to the C.N.S. in poisoning by OHP in more acute exposures. Further detailed examination of C.N.S. changes in a longer series of experimental animals now in progress provides confirmatory evidence that successive exposures to OHP cause irreversible damage of degenerative character in tissue of the C.N.S., especially in nerve fiber tracts, and that it is this damage which is responsible for the chronic motor disability observed in animals following successive exposure to OHP.

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[1] Bean, J. W., and Siegfried, E. C., *Fed. Proc.*, 1943, **2**, 2.

[2] Smith, L., *J. Physiol.*, 1899, **24**, 19.

[3] Shilling, C., and Adams, B. H., ………… …………
1931, **31**, 112.

It has long been known that exposure to OHP causes congestion in various organs of the body, but the finding of especial interest here is that of the injury to the parenchyma of the C.N.S. The occurrence of this tissue damage in animals suffering chronic neuromuscular disabilities as a result of exposure to OHP together with the likelihood that such tissue injury is an extension of some of those etiological factors responsible for the acute after-effects suggest that some of the acute reactions themselves are manifestations of less severe and reversible tissue injury.

The cause of the tissue injury, regardless of its severity and degree of reversibility, must lie in some transient influence. This may well be a combination of the increased CO_2 tension in the tissues, the accompanying increased tissue Ch and a direct action of OHP on cellular enzyme mechanisms, all of which conditions obtain in exposures to O_2 at high pressure. The postulation of a special toxic substance and its persistence after the return of the animal to atmospheric pressure not only lacks experimental support[2,4,5] but also appears to be unessential to explain the reactions which occur during and after exposure to O_2 at high pressure. But in any case the observation that exposure to OHP induces demonstrable tissue injury within the C.N.S. seriously questions the contention that the nervous manifestations of poisoning by OHP are entirely "functional."

The author wishes to express his appreciation to Drs. Wanstrom and Parsons, Department of Pathology; Dr. Loewenberg of the Neuropathological Institute, and to Dr. Crosby of the Neuro Anatomy Department for their help in the examination of the tissues concerned in these experiments.

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[4] Campbell, J. A., *Brit. J. Exp. Path.*, 1937, **18**.

[5] Bert, P., *La Pression Barometrique*, G. Masson, Paris, 1878.

14825

Development *in vitro* of Penicillin-Resistant Strains of the Gonococcus.*

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It has been demonstrated that certain strains of the staphylococcus, the streptococcus, and the pneumococcus acquire resistance to penicillin when grown in broth containing increasing concentrations of the drug. Abraham and his colleagues[1] were able in 16 weeks to adapt a strain of *Staphylococcus*

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* This study was financed by a grant from the Ortho Research Foundation, Linden, N.J.

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

aureus to grow in a concentration of penicillin one thousand times greater than the original inhibitory concentration. McKee and Rake[2] doubled the penicillin-resistance of 2 cultures of sulfonamide-resistant pneumococci after a few daily subcultures. McKee and Houck[3] markedly increased *in vitro* the resistance to penicillin of 3 strains of staphylococci, a pneumococcus, type III, and a strain of *Streptococcus pyogenes*. Schmidt and Sesler[4] were able by serial mouse passage and by serial transfer in liquid medium to develop a penicillin-resistant strain of the pneumococcus. *In vivo* resistance to the drug was also reported by Rammelkamp and Maxon[5] who recovered resistant strains of staphylococci from patients treated with penicillin. With regard to the gonococcus, Frisch[6] has recently reported that by employing chocolate agar as a basic medium he was unable to develop penicillin-resistant strains.

Although penicillin is an effective chemotherapeutic agent in gonococcal infections, a few cases resist treatment. This evidence suggests that either penicillin-resistant strains of the gonococcus occur in nature or the organism acquires resistance to penicillin *in vivo*. The present study was undertaken to determine if the gonococcus when grown in the presence of gradually increased concentrations of penicillin acquires resistance *in vitro*.

Technic. Five strains of *Neisseria gonorrhoeae*, non-resistant to sulfanilamide and sulfathiazole, were selected for the study. They are identified as No. 515, No. 3215, No. 5609, No. 5723, and No. 8071. Three of the strains were isolated from patients with cervicitis and 2 from urethritis in the male. Douglas's broth with 0.05% potassium nitrate, 0.04% potassium dihydrogen phosphate, and 5% lapine blood was used as the basic medium. From 0.005 to 0.01 Oxford unit of penicillin† per ml of the medium was then added because previous tests demonstrated that growth of the selected strains was not inhibited in these concentrations. The cultures were transferred to the same and to a greater concentration of the drug every 48 hours, and sub-cultures for viability were made on chocolate agar plates at the same interval but on alternate days. At approximately weekly intervals, each strain was tested on a suitable carbohydrate medium to determine if any change in fermentation reactions had occurred. Two of the strains which were most resistant to penicillin *in vitro* were then maintained on penicillin-free chocolate agar and also lyophilized by the technic of Flosdorf and Mudd[7] to determine if the acquired resistance to the drug was transient or a permanent factor.

Results. After 32 weeks' exposure to gradually increased concentrations of penicillin in blood broth, 5 strains of *Neisseria gonorrhoeae* grew readily in concentrations 8, 64, 128, 200, and 400 times greater, respectively, than the initial concentrations permitting growth (Fig. 1). The degree of resistance of the two most resistant strains was maintained after cultivation on penicillin-free chocolate agar for 3 weeks. These same strains likewise maintained their resistance after lyophilization for 2 months.

Of special interest were changes in the morphology of the gonococcal cells which were most marked when the organisms were first exposed to the lower concentrations of penicillin. Films from 2 of the strains stained by Gram's method showed groups of enlarged, indistinct, irregularly stained, pleomorphic cells. The cells of the remaining 3 strains were typically biscuit-shaped, but from 4 to 5 times larger than the normal cell. After resistance was acquired, cells conforming in size and shape to a typical gonococcus reappeared. The fermentation of glucose by strains showing pleomorphic cells was delayed.

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[2] McKee, C. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 275.

[3] McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

[4] Schmidt, L. H., and Sesler, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 353.

[5] Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

[6] Frisch, M. C., Behr, B., Edwards, R. B., and Edwards, M. W., *Am. J. Syph., Gonor., and Ven. Dis.*, 1944, **28**, 627.

† Penicillin (Pfizer) was supplied by the Committee on Medical Research of the National Research Foundation, Washington, D.C.

[7] Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.

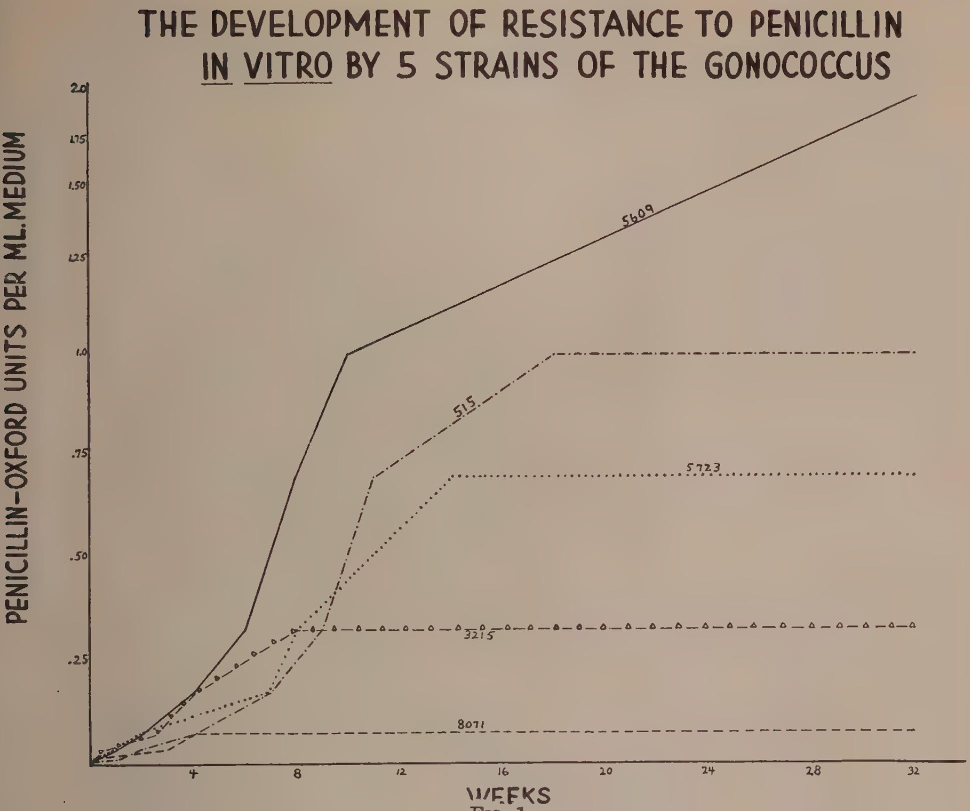

FIG. 1.

The reaction took place from 24 to 48 hours after that observed in the control cultures.

Discussion. The gonococcus is unusually sensitive to penicillin *in vitro*. Cohn and Seijo[8] reported that a concentration of 0.008 Oxford unit per ml of medium was bactericidal for 45.5% of 82 sulfonamide-resistant and non-resistant strains of the gonococcus and that none of the strains would grow in 0.16 unit per ml of medium. The 5 strains which we have studied varied considerably in their adaptability to growth in penicillin. Only comparatively low concentrations of the drug were tolerated. To date the maximum amount of penicillin in which the most resistant strain will grow is approximately 2 units per ml of blood broth. The acquisition of resistance to the drug by the gonococcus was anticipated, however, especially since development of resistance to penicillin by other species of bacteria has been reported.

Morphological changes in bacteria produced by the action of penicillin have been noted by Gardner,[9] who observed an enlargement of cocci in the presence of a bacteriostatic concentration of penicillin. Spink, Ferris, and Vivino[10] reported as permanent characteristics peculiar morphological forms in stained films of *Staphylococcus aureus* during the stage of acquiring resistance to penicillin. Our studies with the gonococcus indicate these morphological changes are temporary.

In their original report on penicillin, Abraham and his colleagues[1] observed that a strain of *Staphylococcus aureus* having developed resistance to penicillin required 48 hours to complete the fermentation of lactose, whereas the control strain effected the same result in 24 hours. A similar delay was noticed in the fermentation of glucose by the penicillin-resistant strains of the gonococcus.

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[8] Cohn, A., and Seijo, I. H., *J. A. M. A.*, 1944, **124**, 1125.

[9] Gardner, A. D., *Nature*, 1940, **146**, 837.

[10] Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

Far more difficulty was encountered in developing penicillin-resistant strains than sulfathiazole-fast strains of the gonococcus *in vitro*. These observations suggest that penicillin-resistant strains of the gonococcus are less likely to become established in the population at large than sulfathiazole-resistant strains, although this possibility should be given consideration.

Summary. Five strains of *Neisseria gonorrhoeae* were made resistant *in vitro* to concentrations of penicillin 8, 64, 128, 200, and 400 times greater than the initial concentration permitting growth. Morphological changes of the gonococcus and a delayed fermentation of glucose were observed in the strains during the development of resistance to the drug.

14826

Action of Certain Sulfonamides on Anaerobic Activity of Respiratory Center of Young Animals.

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Earlier reports have shown that the anaerobic activity of the respiratory center of young animals, as indicated by the gasping pattern of the ischemic or completely isolated head, is altered by various anesthetic agents and tissue poisons[1] and by the carbohydrate stores of the nervous tissue.[2,3] Glucose greatly prolongs survival and the number of gasps of the second series. Insulin and iodoacetic acid diminish or completely abolish the second series: cyanide diminishes the first.[4] These results suggest that the prolonged survival of the respiratory center of young animals is due to an anaerobic source of energy resulting from the breakdown of carbohydrates by enzymes.

Because the sulfa drugs are known to interfere with certain enzymatic processes, it was thought desirable to study the action of these on the gasping pattern and survival of the unperfused isolated head of the young animal. The respiratory center of such a preparation, unlike that of the whole animal with circulation intact, cannot be influenced secondarily by changes in other parts of the body.

Methods. Employing a technic previously described,[1,4] rats 12-15 days of age and weighing 18-26 g were used as test animals. The gasping pattern of the isolated head of rats of this age consists of 28-35 mandibular movements lasting approximately 5 minutes and is composed of an initial or aerobic series having a relatively constant number of gasps, averaging about 9, and a second or anaerobic series having a somewhat variable number (19-26) averaging about 23. A pause or period of complete inactivity between the end of the first series and the beginning of the second (the interseries interval) lasts 35 to 50 seconds. In previous experiments on 65 litters comprising 445 animals, the standard deviation of the mean number of gasps for untreated littermates was found to be slightly less than 2; the average variation for survival was 8.4% and that for number of gasps of the second series was 6.1%.

The drugs employed were sulfanilamide and the sodium salts of sulfathiazole, sulfadiazine, and sulfamerazine. These were injected intraperitoneally in doses of 3, 4.5, and 6 mg. Toxic

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[1] Selle, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 50.

[2] Selle, W. A., *Am. J. Physiol.*, 1944, **141**, 297.

[3] Hiestand, W. A., Tschirgi, R. D., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 153.

[4] Selle, W. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 291.

TABLE I.
Influence of Certain Sulfa Drugs on Survival of Isolated Head of 12-15-day-old Rats.

			Increased respiratory activity based on avg control values					
			After 1 hr		After 2 hrs		After 3 hrs	
Drug	Dose, mg	No. animals	No. anaerobic gasps, %	Total survival %	No. anaerobic gasps, %	Total survival %	No. anaerobic gasps, %	Total survival %
Sulfanilamide	3.0	10	19	29	48	59	39	47
	4.5	12	31	42	61	69	48	62
	6.0	12	39	45	72	78	62	54
Sodium Sulfathiazole	3.0	12	16	22	42	48	32	43
	4.5	10	26	33	55	49	40	51
	6.0	10	34	38	60	66	53	50
Sodium Sulfadiazine	3.0	8	2	0	3	2	4	0
	4.5	10	2	—4	6	3	6	4
	6.0	10	4	8	8	6	4	5
Sodium Sulfamerazine	3.0	8	0	0	—3	—4	0	0
	4.5	10	2	6	4	3	4	8
	6.0	8	4	7	2	7	3	7

manifestations were not observed with the doses employed. Control animals were given an amount of vehicle (.9% NaCl) equal to that used in the treated animals. Following intervals ranging from ¼ to 18 hours, the head was quickly isolated and the ensuing gasps were mechanically recorded by a technique previously described. Deviations from control values were expressed in percent.

Results and Discussion. The results for periods 1 to 3 hours after injection (Table I) show that a significant increase in the total survival time and in the number of gasps of the anaerobic series followed the injection of sulfanilamide or sodium sulfathiazole. Although the total expenditure of energy was not accurately determined, it is apparent that the overall energy liberation for both of these drugs was definitely increased. Neither sodium sulfadiazine nor sodium sulfamerazine was similarly effective in doses equal to those employed for sulfanilamide or sodium sulfathiazole. While the chief effect of the latter drugs was on anaerobic activity, aerobic series being entirely unchanged, the interseries interval was frequently prolonged (average about 35%). Periods less than 60 minutes between injection and decapitation had little or no influence on the gasping pattern; likewise periods greater than 12 hours were without demonstrable effects. Accessory gasps, which appeared in the middle and last third of the anaerobic series, were partly responsible for the marked increase in the total number of gasps. Larger doses (8-10 mg) frequently increased the number of accessory gasps without increasing further the duration of survival.

Although rhythmic, the accessory gasps were smaller and 4-5 times more frequent than the normal or dominant ones. Occurring between the regularly spaced dominant gasps, they produced a striking staircase pattern, which was of two types. In a majority of cases they showed a progressive increase in amplitude until a gasp of normal size occurred, after which this pattern was repeated 3 to 5 times. Contrasted to this "step up" type of accessory gasping was a less frequently occurring "step down" type—in which there was a gradual decrease in amplitude until a dominant spike occurred; as in the other type, the pattern was repeated several times. The accessory gasps of either type were out of phase with or independent of the dominant gasps, which usually were more widely spaced than normal.

The accessory gasps may be due to multiple ectopic pacemakers, or to recirculation of excitatory waves over one or more pathways by a process analogous to that involved in the circus movements of the heart. Such changes in impulse formation or conduction probably represent an imbalance between the various substrates and enzymes implicated in the release of energy through glycolysis, which is known to proceed over an intricate chemical pathway involving numerous stages. High concentrations of the effective sulfa drug may alter impulse initiation or propagation within the reticular gray matter of the medulla by disturbing the enzyme-substrate relationship in one or more stages, and in this way modify the development of the unstable chemical-nervous state necessary for "toppling."[5]

It is not clear why the drugs increase the anaerobic survival. It is possible that depression of certain enzymes diminishes the rate of release of anaerobic energy and thus permits continued utilization of a limited amount of available fuel. However, the apparent increase in overall energy liberation would seem to speak for an enhancing action of certain enzyme systems and an increase in utilizable fuel.

Nor is it clear why sulfanilamide and sodium sulfathiazole were effective in producing the results described while equivalent amounts of sodium sulfadiazine and sodium sulfamerazine were not.

Summary. Sulfanilamide and sodium sulfathiazole in non-toxic doses prolonged the survival of the anaerobic activity of the respiratory center of young rats and increased the overall energy release. Neither sodium sulfadiazine nor sodium sulfamerazine acted similarly.

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[5] Bard, P., *Macleod's Physiology in Modern Medicine*, C. V. Mosby Co., St. Louis, 1941, p. 555.

14827

Recovery of Creatinine After Ingestion and After Intravenous Injection in Man.

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It is a known fact that not all the creatinine administered by mouth is excreted in the urine.[1] The amount so excreted is at most 80% of the quantity ingested and is usually less. Although no satisfactory explanation has been offered for this incomplete recovery, creatinine is regarded by many as a waste product of metabolism.[1,2] The possibility that a small amount of creatinine may be destroyed by intestinal bacteria has been mentioned by some investigators.[3] From the fact that creatinine is not stored in the tissues, these same investigators conclude that such creatinine as is not excreted undergoes some transformation in the body.[3] This conclusion, however, is not valid unless it is proved that creatinine is completely absorbed by the intestine. The possibility that part of the ingested creatinine may not be absorbed has received practically no consideration.

We have studied the recovery of administered creatinine in several human subjects. One of these subjects (G.N.B.), as treatment for a chronic ulcerative colitis complicated by hemorrhages, had a permanent ileostomy near the cecum. This operation severed the continuity of the intestine. At the time of the experiments, the fistula was working well, hemorrhages had ceased and the man was in apparent health. Another subject (W.K.) was studied on 3 occasions. At the time of the first 2 experiments he was in good health, but at the time of the last experiment he was greatly emaciated because of a carcinoma of the esophagus. The other subjects were either volunteers in perfect health or ambulatory

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[1] Hunter, A., *Physiol. Rev.*, 1922, **2**, 586.

[2] Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, 1939, **131**, 111.

[3] Beard, H. H., *Creatine and Creatinine Metabolism*, Chemical Publishing Company, Inc., Brooklyn, N.Y., 1943.

TABLE I.

Recovery of Creatinine Administered to Normal Subjects and to One with an Ileal Fistula.

				Creatinine excreted in stated time				
					From urine			
Subject	Sex	Age yr	Creatinine given by mouth (M) or by vein (V) g	From fistula g	Total g	Endogenous* g	Exogenous g	% recovered
M.B.	F	25	6.0 M		5.0 (23.1 hr)	1.2	3.8	63
S.S.B.	M	35	8.0 M		6.7 (25.2 '')	1.9	4.8	60
A.P.	F	27	8.0 M		6.6 (23.6 '')	1.7	4.9	61
B.G.	M	31	10.0 M		9.2 (24.4 '')	1.9	7.3	73
W.K.	M	55	12.0 M		8.1 (24.5 '')	1.0†	7.1	59
		50	15.0 M		9.2 (24.5 '')	1.7	7.5	50
			4.8 V		6.2 (25.6 '')	1.7	4.5	94
V.R.	F	26	9.5 V		11.7 (27.9 '')	1.7	10.0	105
			20.7 V		22.0 (26.4 '')	1.6	20.4	98
			23.3 V		22.7 (27.7 '')	1.7	21.0	90
H.G.	M	56	12.6 V		13.4 (27.7 '')	1.6	11.8	94
J.J.	M	53	16.2 V		17.0 (24.1 '')	1.6	15.4	95
G.N.B.§	M	24	4.0 M	1.0 (6.4 hr)	2.4 (6.4 '')	.38	2.0	94‡
			8.0 M	1.9 (10.0 '')	8.0 (24.0 '')	1.4	6.6	106

* The rates of the endogenous creatinine output of these subjects, in the same order of the table, are: 50, 74, 73, 78, 68, 61, 59, 66 and 60 mg/hr. The mean of these rates is 65.4 and the standard error of the mean is 2.94. Three times this standard error represents 13.4% of the mean. We have verified that a variation of this magnitude in the outputs of endogenous creatinine in these experiments changes by 3.8 the average % recovered after ingestion and by 2.2 the average % recovered after intravenous injection.

† At the time of this experiment on W.K. the excretion of endogenous creatinine had fallen to 43 mg per hr. This subject was then suffering from a carcinoma of the esophagus and was considerably emaciated.

‡ We do not have a 24-hr collection in this experiment because the subject failed to follow instructions. An estimate of the amount of exogenous creatinine recovered in 24 hr can be obtained as follows. The amount recovered in about 6 hr (from 6.0 to 6.3 hr) in the other ingestion experiments was, in the order of the table, 2.9, 3.9, 3.3, 5.3, 4.9, and 5.5 g and in the second experiment on G.N.B., 4.3 g. The ratio of each of these figures to the corresponding exogenous amount excreted in about 24 hr (column headed ''Exogenous'') is, in the same order, .763, .812, .674, .726, .690, .733, and .652. The mean of these ratios is 0.721. Applying this mean to the data of the first experiment on G.N.B., we get for the amount recovered in 24 hr, 2.0/.721 = 2.77 g. This amount added to the amount from the fistula gives 3.77 g, or 94% of the quantity ingested. This is the % shown in the table.

§ These experiments could not be repeated because this subject developed a fatal subacute bacterial endocarditis.

patients with minor ailments. None had any evidence of renal disease.

The endogenous excretion of creatinine was examined at irregular intervals of time on different days and in different parts of the day and night, and the average hourly rate computed. The creatinine concentration in the urine was determined by the method of Folin.[4] During the control periods and during the experiments, the subjects were up and about in the laboratory and followed their customary diets.

The age, sex, quantity of creatinine given, mode of administration and amount excreted in the urine are shown in Table I. The rates of endogenous creatinine excretion are given in a footnote of Table I. For the estimation of the endogenous amounts excreted during the experiments these rates were treated as constants.

Corroborating previous workers, we found that the amount of creatinine recovered in the urine within 24 hours after ingestion varies between 50 and 73% of the quantity ingested (Table I). In our other experiments in which the collection of urine was not carried out for 24 hours, the amount recovered in 24 hours was calculated by fitting exponentials to the latter part of the data and integrating. In 13 of the latter experiments in 10 subjects, the amount actually recovered in 6.5 hours was 44% and the average 24-hour total calculated by integration was almost 60% of the quantity ingested. In 6 other experiments in one subject,[5] the average amount actually recovered in 8.1 hours was 60% and the average 24-hour total calculated, 70% of the quantity ingested.

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[4] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

When creatinine was injected intravenously the results were entirely different. On an average, 96% of the quantity injected was recovered in about 24 hours (Table I). Incidentally, this percentage in human experiments is about equal to the percentage obtained after intravenous injection in the dog.[6] In intravenous experiments, therefore, there is no evidence of creatinine transformation.

In order to ascertain whether the smaller percentage of the creatinine excreted in oral experiments was the result of incomplete absorption, we gave creatinine to the subject with the fistula (G.N.B.) and determined both the amount of creatinine collected from the fistula and the amount excreted in the urine. The experiments were carried out after we had convinced ourselves that creatinine added to the material from the fistula could be recovered quantitatively by the method of Folin.[4] Blank analysis of this material gave a color development equivalent to a creatinine concentration of 2 to 7 mg per 100 cc. Expressed in terms of creatinine, the rate at which these chromogenic substances were collected from the fistula was, on an average, 5 mg per hour. This small amount was subtracted from the total amount collected in actual experiment.

The results of 2 experiments after ingestion of 4 and 8 g, respectively, are given in Table I (subject G.N.B.). Creatinine began to appear in the fluid from the fistula during the second hour after ingestion and continued to appear from the second to the fifth hour, with a peak during the third hour. The total amount of creatinine collected from the fistula represents 24 to 25% of the quantity ingested (Table I).

In oral experiments, therefore, creatinine is not completely absorbed by the small intestine. That it is probably not absorbed by the colon follows from the fact that, although in subject G.N.B. the ingested creatinine could not reach the colon, this subject excreted in the urine 81% of the quantity ingested, an amount of creatinine which is as large as the largest to be expected in normal subjects.

Within the limits of experimental error, the amount of creatinine excreted in the urine was equal to the difference between the quantity ingested and the amount collected from the fistula. Hence, the amount excreted in the urine represents the amount actually absorbed. There is no need for assuming a transformation of creatinine to account for the whole quantity ingested.

The recovery of creatinine after administration by routes other than oral and intravenous deserves re-investigation.

Summary and Conclusions. In a patient with an ileal fistula it was shown that about 25% of ingested creatinine was expelled unabsorbed from the fistula. The amount of creatinine excreted in the urine was equal to the amount absorbed. The evidence suggests that, in oral experiments, no creatinine is absorbed by the large intestine. When injected intravenously, creatinine is virtually all excreted in the urine. These results are at variance with the hypothesis of creatinine transformation in the human body.

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14828

Decomposition Products of Nucleoproteins and Related Substances and Muscle Sensitivity to Acetylcholine and Potassium.*

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In the search for substances with curare-like effect and substances that increase the sensitivity of the effector cells to acetylcholine the effect of decomposition products of nucleoproteins and related substances on the sensitivity of striated muscle to acetylcholine and potassium were investigated.

Nucleic acid and some decomposition products thereof (adenylic acid, guanylic acid, cytidylic acid, inosinic acid, adenosine, adenine, guanine, guanosine, inosine) and methylxanthines (caffein, theobromine, theofilline) may induce a vasodilatation,[1-11] may modify the contraction of the heart, striated and smooth muscle,[1,12-17] and methylxanthines and guanidine may induce a contracture of the striated muscle.[18] It is likely that these effects are independent of the action of acetylcholine-like substances liberated at the synapses since the above substances exert their effect even after administration of atropin.

Method. The rectus abdominis muscle of frog was excised and suspended in a muscle chamber containing 10 cc of Ringer's solution. The Ringer's solution was changed to an acetylcholine solution (50 μg acetylcholine per 100 cc Ringer's solution) for 2 minutes. The muscle was then washed with Ringer's solution for 10 minutes. This procedure was repeated until 3 successive exposures to the solution of acetylcholine gave similar responses. Afterwards the muscle was washed only for 5 minutes and immersed in one of the solutions of the substances used. A series of solutions containing each substance in increasing concentrations was used. The pH of all solutions used was corrected to 7. The height of contraction of the muscle was registered by an isotonic lever on a kymograph. The tracings were measured, the amount of contraction of the rectus abdominis muscle before application of the substances was taken as 100% and the amount of contraction after application of the substances was expressed as percent of it.

In another series of experiments a 20 mM solution of potassium chloride in Ringer's solution was used instead of acetylcholine.

Results. The effects of the substances used on the height of contraction of the rectus abdominis muscle induced by acetylcholine and potassium are given in Table I. Within

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* This study was aided by a grant from the John and Mary R. Markle Foundation.

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TABLE I.
Effect of Substances on the Contraction of the Rectus Abdominis Muscle Induced by Acetylcholine or Potassium.

	Magnitude of contraction in % of control.* Contraction induced with:									
	Acetylcholine					Potassium				
	Concentration of the substances used (mg per 100 cc Ringer's solution):									
Substance	200	20	2	0.2	0.02	200	20	2	0.2	0.02
Nucleic acid (yeast)		82	80	100	102		75	78	80	78
Adenylic acid	105	102	102	102	100	171	143	138	125	105
Adenosine		99	98	98	102		135	125	112	113
Adenine sulfate		109	107	105	100		138	117	109	106
Adenine acetate		102	102	102	99		97	116	110	102
Guanine		107	110	104	101		228	185	161	146
Xanthine	113	114	105	106	101	175	162	152	148	127
Uracil	138	105	103	98	97	209	182	162	167	135
Thiouracil	175	106	105	101	97	258	170	140	126	113
Uric acid	105	102	99	105	105	224	150	150	142	124
Caffeine		299	123	96	95		281	157	124	122
Theobromine		182	107	93	93		253	160	118	114
Theofilline		152	102	98	104		348	156	128	110
Pyridine		151	101	100	100		180	133	118	115
2-methyl-5-ethoxymethyl-6-amino pyrimidine	340	136	105	100	98		172	127	107	111
4-methyl-5-β-hydroxyethyl thiazole	336	137	100	100	100		170	122	120	109
Pyrol		142	93	86	89		305	197	132	125
Piperidine		71	98	95	100		88	100	107	110
Alloxan	80	92	93	98	104	47	104	103	103	102
Methylguanidine		118	105	103	101		267	120	113	117
Urea	82	100	99	103	104	102	146	137	129	128
Thiourea	84	89	90	90	98	89	153	126	132	112

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than ±5%.

the range of concentrations used the effect of acetylcholine was either not modified (uric acid, adenosine, adenine, guanine, adenylic acid), or was not modified in low concentrations and was increased in higher (methylxanthines, uracil, thiouracil, methylguanidine, xanthine, pyridine, pyrol, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, 4-methyl-5-β-hydroxyethyl thiazole), or was not modified in low concentrations and decreased in higher (alloxan, nucleic acid, urea, thiourea, piperidine).

The effect of potassium was either increased (uric acid, methylxanthines, uracil, thiouracil, urea, thiourea, adenosine, adenine, adenylic acid, guanine, xanthine, methylguanidine, pyridine, pyrol, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, 4-methyl-5-β-hydroxyethyl thiazole) or decreased (nucleic acid, alloxan, piperidine).

The amount of contraction induced by potassium cannot be compared to that induced by acetylcholine since these substances cause different physicochemical changes in the muscle cells.[18,19] However, substances changing the amount of contraction induced by acetylcholine usually have similar effect on the muscle contraction following stimulation of the motor nerve and usually modify the activity of choline esterase. Substances changing the height of contraction induced by potassium usually modify the effect of other chemical agents that are able to induce muscle contraction by direct contact with the striated muscle.

Summary and Conclusions. 1. The effect of decomposition products of nucleoproteins and related substances on the contraction of the rectus abdominis muscle induced by acetylcholine and potassium was studied. 2. Adenosine, adenine, adenylic acid, and uric acid did not modify the effect of acetylcholine. 3. Xanthine, methylxanthines, uracil, thiouracil, pyridine, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, 4-methyl-5-β-hydroxyethyl thiazole, methylguanidine, and pyrol did not modify the

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effect of acetylcholine when used in low concentrations. They increased the effect of acetylcholine when present in higher concentrations. 4. Nucleic acid, alloxan, urea, thiourea, and piperidine decreased the effect of acetylcholine when used in higher concentrations. 5. Adenosine, adenine, guanine, xanthine, adenylic acid, uric acid, methylxanthines, uracil, thiouracil, pyridine, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, 4-methyl-5-β-hydroxyethyl thiazole, thiourea, urea, methylguanidine, and pyrol increased the effect of potassium in inducing muscle contraction. 6. Nucleic acid, alloxan, and piperidine decreased the effect of potassium in inducing muscle contraction.

The authors wish to express their gratitude to Merck & Co., Inc., for the generous supply of 2-methyl-5-ethoxymethyl-6-amino-pyrimidine and 4-methyl-5-hydroxyethyl thiazole.

14829 P

Effect of Alloxan on Carbohydrate and Uric Acid Metabolism of the Pigeon.*

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Alloxan in a dose of 150 to 200 mg per kg injected intravenously into a pigeon causes an elevation of the blood sugar from the normal level of about 150 mg% to values between 300 and 400 mg% within 24 to 48 hours. One day after the injection the animals appear very sick; they are weak and drowsy, sit with their eyes closed, make no attempt to move or fly and usually die in this stupor within 2 or 3 days. Smaller doses of alloxan (60-125 mg per kg) may cause a similar syndrome except that the blood sugar may remain within the normal range. A disturbed carbohydrate metabolism can therefore not be the only cause of this condition.

At autopsy such pigeons present a very unusual picture. All serous membranes, especially the pericardium, the pleura, and the surface of the liver are covered with a thin whitish layer which at first glance may look very much like fibrin as seen in polyserositis. This material, however, can easily be removed from the membranes, and identified chemically and microscopically as sodium urate. In one instance 8 mg of sodium urate were collected from the pericardium and the liver surface alone.

Histological examination of the kidneys reveals massive infiltration with sodium urate which is deposited mainly in the greatly dilated tubules, but also in the interstitial tissue. Smaller collections of crystals are found on the serosal surfaces and occasionally in the parenchyma of the liver. Evaluation of the changes in the pancreatic islets is very difficult because even the normal pigeon pancreas may contain extremely few beta cells. However, in some cases which showed hyperglycemia no beta cells could be found at all, or the occasional cells found looked profoundly degranulated. In other cases the islets were normal.

In view of these findings, it seemed of importance to study the effect of alloxan on the uric acid metabolism of pigeons. A series of more than 30 pigeons was injected with various doses of alloxan, and the blood uric acid level was determined before and at intervals after the injection. Folin's[1] method for the estimation of uric acid was used. Blood samples were taken from the wing vein. The normal blood uric acid content of our pigeons

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TABLE I.

Alloxan mg/kg	Before	Blood uric acid mg per 100 cc Hr after injection 6	24	48	72	96
60	5.2	11.0	40	18.3	10.5	8
75	7	34	135	died	—	—
75	6	8	28.3	14.3	8.5	10.0
75	5.4	9.3	9.3	8	6.5	6.0
75	4.3	8	35.5	11	8	5.1
125	4.2	9.2	90	weak	killed	—

ranged between 4 and 8 mg%. About one-third of the birds maintained a normal blood uric acid level after the injection of alloxan, and exhibited none of the symptoms described in the others, however, symptoms developed and the uric acid content of the blood increased markedly, reaching its peak about 24 hours after injection. The birds usually died within 48 hours if blood levels higher than 60 mg% were reached; if the peak was lower, they recovered slowly from their weakness and drowsiness and appeared normal from the third or fourth day on. At the same time the blood uric acid level returned to normal. Table I illustrates the course of the blood uric acid level in 6 pigeons over a period of 4 days.

The serous membranes of pigeons which had survived the alloxan injection and whose blood uric acid levels had returned to normal showed only very slight deposits of sodium urate or none at all. The kidney parenchyma appeared normal. An infiltration with uric acid, if it had occurred, must have been transitory and the sodium urate was excreted. The 24-hour excretions of 10 pigeons, which during the experiment had been placed in individual metabolism cages, were analyzed for their pyrimidine content. Though these estimates were rather crude, the increase in blood uric acid was apparently accompanied and followed for a few days by an increased excretion of pyrimidine compounds. Some of the birds which had apparently recovered later became weak again, and died after 2 or 3 weeks in a state of emaciation, the most striking autoptic finding being a marked muscular atrophy, especially in the breast muscles. Microscopically, there were severe degenerative changes, ranging from waxy homogenization and vacuolation to complete breakdown and disappearance of the fibers. Sarcolemma nuclei were very numerous and in addition there were small lymphocytic infiltrates scattered throughout the interstitial tissue.

After our first observations were made, we learned that a similar syndrome has been produced in fowls by feeding large amounts of protein; it is known to the zoologist as "visceral gout."[2] The protein fraction responsible for the condition seems not to have been determined.

Among the compounds related to alloxan, barbituric acid, uracil, and violuric acid did not change the blood uric acid level, but dialuric acid, the reduction product of alloxan, proved to be just as effective as alloxan on the uric acid metabolism of the pigeon. This is surprising since it does not have an alloxan-like effect on the islet cells. The components of dialuric acid, urea and tartronic acid, injected separately, were both ineffective.

Summary. Alloxan, injected into pigeons in a single dose of 60 to 125 mg/kg (less than one-half of the diabetogenic dose) sometimes causes a marked elevation of the blood uric acid level and a deposition of sodium urate in the tissues. A conspicuous atrophy of the musculature is observed as a late effect in pigeons which recover from the acute stage of alloxan poisoning. Among several compounds, related to alloxan, only dialuric acid was found to possess a similar effect.

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14830

The F_2 Excretion of Normal Men on Different Levels of Niacin Intake.*

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Najjar and his collaborators[1,2] reported the presence in urine from normal individuals of a fluorescent substance which they called F_2. This compound was excreted in fairly large amounts when the niacin intake was high. In cases of pellagra the excretion of F_2 was diminished or even zero in some cases. When large amounts of niacin were ingested by normal individuals or during the course of pellagra therapy, the F_2 excretion increased. Their evidence indicated that this fluorescent substance was related to the metabolism of nicotinic acid.

Recently both the Johns Hopkins group[3,4] and Huff and Perlzweig[5] have shown by means of chemical isolation that the fluorescent substance is a derivative of niacin, suggesting that F_2 is N-methylnicotinamide or some compound closely related to it.

A description of the method for the determination of this substance was published by Najjar and coworkers.[6] Later a modified procedure was proposed by Huff and Perlzweig.[7] Recently Najjar published another modification.[8] Some of our results with these procedures have been reported in a preliminary note.[9]

Experimental. As a part of a comprehensive study of the B vitamins[10] 4 normal young men were maintained for a period of 161 days on a diet which supplied an average of 12.4 mg of niacin per day. During the same period 4 other men were given the same diet plus an additional 10 mg of niacin. This was followed by a period of 32 days when the niacin intake in the diet was reduced to 0.12 mg per day. The niacin in the diet was determined by the microbiological method.[11] Twenty-four-hour urine samples were collected throughout the period at intervals of several days. These samples were preserved by adding 5 ml glacial acetic acid and 5 ml toluol to the collection bottles. In most cases the analyses were made on the same day the collection was completed.

Throughout the first dietary period, the urines were analyzed by the original method of Najjar, *et al.*[6] This method was slightly modified to permit the determination of thiamine and F_2 on the same eluate from the zeolite column. When the severely restricted diet was started, the method of Huff and Perlzweig[7] was used. Najjar informed us of his modified procedure[8] after all of these analyses were completed.

The procedures of Najjar *et al.*[6] and Najjar[8]

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TABLE I.
Comparison of F_2 Excretions by the Old[6] and New Methods[8] of Najjar.

All values expressed in mg N-methylnicotinamide chloride per 100 cc urine and as percentages of the means for the corresponding methods.

	Old method		New method	
Subject	mg	% of mean	mg	% of mean
Wa	0.378	45.5	0.252	40.1
Jo	0.489	58.9	0.318	50.6
Gl	0.567	68.3	0.441	70.1
Se	0.630	75.9	0.511	81.2
Ja	0.674	81.2	0.584	92.8
Ph	0.882	106.3	0.696	110.6
Oa	1.45	174.7	1.03	163.8
Ol	1.57	189.2	1.20	190.8
Mean	0.830	100.0	0.629	100.0

were compared with the results shown in Table I. The values in all of our determinations were originally calculated as quinine sulfate equivalents and were then converted to mg of N-methylnicotinamide chloride by multiplying by 35 as suggested by Najjar.[8] The absolute values for F_2 excretion as determined by the two methods differ considerably, but in spite of the wide range, the relative ranking of the men was the same in both cases. For each subject, the differences between the percent of the means is within the experimental error of the methods. Although there are not as many comparisons as would be desired, it is believed they indicate that any marked alterations in F_2 excretion will show up by either one of the methods. Najjar,[8] furthermore, has indicated that the value of his modified procedure resides primarily in overcoming the false zero F_2 excretions on very low niacin intakes and the masking of small amounts of F_2 in fasting one-hour or in casual specimens.

Results. At the start of the controlled dietary intake of niacin, F_2 analyses were made on the 24-hour urine samples collected once each week. Some representative values of the F_2 excretion during this period are given in Table II. After the subjects had been on their respective diets for 3 months, a consideration of the results (Table II) indicated that the method of Najjar *et al.*[6] did not differentiate between the 2 levels of niacin intake. On a number of occasions, even negative values were secured in both groups for the urinary excretion of F_2. These considerations and others led us to abandon this procedure.

The average for the F_2 excretion of the men on the intake of 12.4 mg was 3.22 mg (as N-methylnicotinamide) per day whereas that for the supplemented group was 3.83 mg. There is, however, such great overlapping of the two groups that no acceptable differentiation of the subjects can be made. The averages of all the analyses that were made during this complete period show essentially the same random distribution of the subjects as in Table II.

When the subjects were put on the severely restricted diet, 2 men from the supplemented group and 2 from the restricted group were put on an intake of 0.12 mg niacin while the other 4 were given an additional 10 mg. During this time the urines were analyzed for F_2 by the method of Huff and Perlzweig.[7]

Table III shows the results of the urinary excretion of F_2 of these subjects during the severely restricted regimen. The values are expressed as mg of quinine sulfate since no N-methylnicotinamide was available when the analyses were made. The results indicate that even when the intake of niacin was restricted to 0.12 mg per day for a period of 32 days, the excretion of F_2 showed only a slight and inconsistent decrease as determined by this method. At the end of the period the subjects cannot be divided into their proper groups on the basis of either single excretions or the averages for the entire period.

Discussion. A compound as stable as niacin is not likely to be entirely destroyed by the body but at present it appears that the sum of all recognized end products of niacin metabolism in the urine accounts for no more than 10% of the intake.[7-9] Such evidence as is available from related compounds suggests that the excess of niacin intake over metabolic use may be excreted in a conjugated form. It would seem reasonable to suppose that some compound such as F_2 might be excreted in proportion to the excess of intake over need or, inversely, to the extent of deficiency. The present evidence indicates that this simple

TABLE II.

Excretion of F_2 on Controlled Intakes of Niacin.

24-hour urinary excretion of F_2 expressed as mg N-methylnicotinamide chloride. Only a few representative values are given. The controlled diet was started 6-7-43. The method of Najjar *et al.*[6] was used in these analyses.

Niacin intake	12.4 mg per day					22.4 mg per day				
Subject	Ge	Wi	Tr	Wa	Mean	Se	No	Jo	Ja	Mean
6-24	1.49	2.81	4.20	1.68	2.55	5.30	3.19	0	2.19	3.56
7-19	5.48	3.01	3.52	3.00	3.75	4.57	2.54	3.33	5.01	3.88
7-26	2.89	1.51	2.95	3.06	2.60	1.88	2.91	1.69	4.88	2.84
8-17	1.97	1.82	4.46	4.78	3.26	5.64	5.92	4.76	6.00	5.58
8-30	4.74	5.29	3.18	2.53	3.93	4.66	4.10	2.63	1.82	3.30
Mean	3.31	2.89	3.66	3.01	3.22	4.41	3.73	2.48	3.98	3.83

TABLE III.

Excretion of F_2 on Very Low Intakes of Niacin.

24-hour urinary excretion of F_2 expressed as mg of quinine sulfate. All analyses were made by the method of Huff and Perlzweig.[7] The diet was started November 15 and continued through December 17, 1943.

Niacin intake	0.12 mg per day					10.12 mg per day				
Subject	Ge	Wi	Se	Ja	Mean	No	Jo	Tr	Wa	Mean
11-8	0.11	0.46	0.69	1.23	0.62	0.29	0.84	0.46	0.87	0.62
11-15	1.11	0.64	0.70	0.96	0.85	0.52	1.56	1.09	1.02	1.05
11-18	0.26	0.16	0.30	0.26	0.25	0.24	0.35	0.41	0.26	0.32
11-21	0.22	0.12	0.16	0.20	0.18	0.32	0.32	0.22	0.20	0.27
12-1	0.25	0.11	0.14	0.25	0.19	0.21	0.33	0.44	0.25	0.31
12-7	0.69	0.70	0.24	—	0.54	0.18	0.25	0.42	0.36	0.30
12-16	0.22	0.32	0.15	—	0.23	0.26	0.35	0.33	0.44	0.35
Mean	0.41	0.36	0.34	0.58	0.41	0.29	0.57	0.48	0.49	0.46

theory does not apply to the rather considerable range of conditions represented in our experiments.

Several possible explanations for the results may be suggested. In the first place the body may require less than 12 mg of niacin per day and all the excess may be eliminated in some form other than F_2 or N-methylnicotinamide. No differentiation would then be expected between 12.4 and 22.4 mg per day but it might still be possible to distinguish between lower levels of intake by measurement of F_2 excretion. Secondly, the metabolic turnover in the body may be so slow that even a substantially zero intake for 32 days would not be reflected in the excretion of substances like F_2. We may note that at no time were there any signs or symptoms in these subjects which could clearly be referred to as a niacin deficiency. Finally, it is conceivable that the particular type of diet used may have altered the bacterial flora of the intestine so that synthesis of niacin from this source obscured the effects of the alterations in intake of niacin. The diet used in the prolonged experiment with niacin at 12.4 and 22.4 mg per day was not remarkable except for a low level of the whole B complex but the diet used for the final 32 days was chiefly of the synthetic type and therefore greatly abnormal.

In any case it appears that measurement of the excretion of F_2, under the conditions applied here, is worthless as a means of evaluating the state of niacin nutrition though it may have value in cases of much more prolonged restriction in intake. Both the stability of niacin and the failure of present methods to account for its disappearance after ingestion argue for continuation of the search for the end products. It may be significant that the excretion of F_2 shows great variations from day to day, even on a rigidly constant diet and with maintenance of standardized physiological conditions including physical activity. These variations are not reflections of careless technic but continue in spite of elaborate efforts to control the methods.

We have had some indication that part of the fluorescence measured as F_2 is related to thiamine; the blank in the thiamine determination, using zeolite adsorption as in the Najjar method for F_2, increases as the thiamine excretion decreases and this is true even though the niacin intake is constant at about 20 mg per day. Time has not permitted us to investigate this question further.

Summary. F_2 analyses have been made on the urine of normal young men who were maintained on controlled niacin intakes. The method of Najjar showed no difference in the F_2 excretion of 2 groups whose intakes over a period of 161 days averaged 12.4 and 22.4 mg of niacin per day. There was no difference in the F_2 excretion of 2 groups maintained for 32 days on intakes of 0.12 and 10.12 mg of niacin per day when the method of Huff and Perlzweig was used for the determination.

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Experiments on Erythrocyte Sedimentation Rate*

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In general the rate of erythrocyte sedimentation (E.S.R.) is considered to be dependent on the nature and concentration of the protein components of the plasma (*lit.*[1]). However there is some experimental evidence suggesting that E.S.R. may likewise be profoundly influenced by non-protein substances. Fahraeus[2] demonstrated that gum arabic added to normal blood caused an increased E.S.R. The chemically closely related type III pneumococcus polysaccharide[3] was shown to have a similar effect.[4] Shedlovsky and Scudder several years ago in an unpublished lecture reported an increase in E.S.R. on the addition of viscous extracts of certain sarcomata which presumably contained hyaluronic acid. Similar experiments were performed by Kendall (personal communication) and by us (unpublished) with pure hyaluronic acid.

In this paper experiments will be presented which, in combination with published evidence, indicate that probably all highly asymmetrical molecules of large size cause an increase of E.S.R. when added to normal blood. Experiments on the intravenous injection of hyaluronate and hyaluronidase are likewise reported.

Experimental. The first experiments deal with the *in vitro* increase of E.S.R. induced in normal human blood by various substances. E.S.R. in all experiments was measured by the Westergren method, making no correction for cell volume or protein concentration. All blood samples were obtained from healthy young males and, after mixing with the additions indicated in the experiments, were incubated for 30 min. at 37°C. The samples were then drawn up in the sedimentation tubes and read every 15 minutes for 1 hour.

The samples of hyaluronic acid used were prepared from cattle synovial fluid,[5] from a

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* This work was supported in part by a grant from the Josiah Macy, Jr., Foundation. Part of these data was reported at the meeting of the American Chemical Society, Biochemical Section, New York, Sept., 1944.

[1] Nichols, R. E., *J. Lab. and Clin. Med.*, 1942, **27**, 1317.

[2] Fahraeus, R., *Acta Med. Scand.*, 1921, **55**, 3; *Physiol. Rev.*, 1929, **9**, 241.

[3] Heidelberger, M., Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **49**, 847.

[4] Nungester, W. J., and Klein, L. F., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 315.

[5] Meyer, K., Smyth, E. M., and Dawson, M. H., *J. Biol. Chem.*, 1939, **128**, 319.

TABLE I.
Effect of Addition of Hyaluronate, Thymonucleate, Gelatin, and Fibrinogen to Normal Blood.

Sample	MM sedimentation			
	15′	30′	45′	60′
2 cc blood + 0.5 cc NaCl	1	2	5	6
’’ + 0.4 ’’ ’’ + 0.1 cc Enzyme C (2 mg)	0.5	1	1	2
’’ + ’’ ’’ highly polymerized hyaluronate (5 mg) + 0.1 cc NaCl	72	117	120	120
’’ + ’’ ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	5	10	13	17
’’ + ’’ ’’ ’’ ’’ ’’ (2 mg) + ’’ ’’ NaCl	29	68	93	113
’’ + ’’ ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	0.5	1	2	3
’’ + ’’ ’’ ’’ ’’ ’’ (1 mg) + ’’ ’’ NaCl	13	35	54	69
’’ + ’’ ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	0.5	1	2	3
’’ + ’’ ’’ depolymerized hyaluronate (5 mg) + ’’ ’’ NaCl	1	5	8	13
’’ + ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	0.5	1.5	2	4
’’ + ’’ ’’ ’’ ’’ (2 mg) + ’’ ’’ NaCl	0.5	2	4	6
’’ + ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	0.5	0.5	2	2
’’ + ’’ ’’ ’’ ’’ (1 mg) + ’’ ’’ NaCl	1	2	4	7
’’ + ’’ ’’ ’’ ’’ ’’ + ’’ ’’ C (2 mg)	0	1	2	2
’’ + ’’ ’’ Thymonucleate (5 mg) + ’’ ’’ NaCl	5	48	90	110
’’ + ’’ ’’ Gelatin (24 mg) + ’’ ’’ ’’	70	112	114	120
’’ + ’’ ’’ ’’ (6 ’’) + ’’ ’’ ’’	17	30	32	41
’’ + ’’ ’’ Fibrinogen (25 mg) + ’’ ’’ ’’	13	45	72	86
’’ + ’’ ’’ ’’ (12.5 ’’) + ’’ ’’ ’’	3	12	20	30
’’ + ’’ ’’ ’’ (5 ’’) + ’’ ’’ ’’	2	5	8	12
’’ + ’’ ’’ ’’ (2 ’’) + ’’ ’’ ’’	1	2.5	4	6

Hyaluronidase even in higher concentrations had no effect on the samples containing thymonucleate, gelatin, or fibrinogen.

human mesenthelioma,[6] or from human umbilical cord.[7] As indicated (Table I), one sample was prepared as the free acid by precipitation from glacial acetic acid.[7] This procedure leads to a depolymerization as expressed by a low viscosity. To make the desired dilution, a weighed amount of the sample was neutralized with the amount of N/10 NaOH determined previously by titration. The other sample was prepared as the sodium salt. After removal of protein by chloroform-amyl alcohol and zinc hydroxide precipitation, the viscous material in 5% sodium acetate was reprecipitated by 1.5 volumes of alcohol to which about 0.5% of glacial acetic acid was added. The fibrous sodium salt was then reprecipitated from sodium acetate solution by alcohol. The purity of the hyaluronic acid samples was checked by analysis for nitrogen, hexosamine, acetyl, uronic acid, and specific rotation.[7]

Sodium desoxyribonucleate was prepared from calf thymus according to Hammarsten.[8]

The gelatin used was a 6% solution in 0.9% sodium chloride (Knox Gelatin Co. Lot No. P x 10-20) "for use as an infusion colloid," containing no preservative.

The sample of human fibrinogen used was prepared in the laboratory of Dr. Edwin J. Cohn. It dissolved easily in water to give a slightly opalescent solution in a concentration of 6%.

The samples of hyaluronidase were prepared from ram or bull testis by a method using ammonium sulfate and lead acetate fractionation, similar to the method of Morgan and

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[6] Meyer, K., and Chaffee, E., *J. Biol. Chem.*, 1940, **133**, 83.

[7] Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

[8] Hammarsten, E., *Biochem. Z.*, 1924, **144**, 383.

McClean.[9] The material must be free of nucleic acid or otherwise an increase in E.S.R. may result. All testis preparations contained, besides hyaluronidase, an enzyme hydrolyzing chondroitinsulfuric acid with the liberation of acetylchondrosaminosulfuric acid-glucuronide. (The action and properties of this enzyme will be reported separately.)

In Table I the effect of the addition of viscous and non-viscous hyaluronic acid, thymonucleic acid, gelatin, and fibrinogen to normal blood is shown, together with the action of testis hyaluronidase. It can be seen that the sample of viscous hyaluronate causes maximal E.S.R. in a concentration of about 0.2%. The increased rate is promptly abolished by incubation with testis hyaluronidase. The non-viscous hyaluronate has no appreciable influence on E.S.R. Thymonucleate about equals the effect of hyaluronate, while the gelatin and fibrinogen samples used had a quantitatively inferior effect, since with the latter much larger concentrations were required to approximate the effect produced by hyaluronate. The specificity of the hyaluronidase was apparent from its failure to decrease the E.S.R. with substances other than hyaluronate. The failure of the testis enzyme preparation to act on the rate increased by fibrinogen is in accord with experiments on the possible proteolytic activity of these testicular extracts. Neither with fibrinogen nor with other proteins could a proteolytic effect ever be detected.

In order to decide what effect hyaluronic acid might have on E.S.R. *in vivo,* 6 guinea pigs and one young rabbit were injected intravenously with hyaluronate in sodium chloride solution. In the guinea pigs the superficial lateral veins of the legs were used. The E.S.R. was determined by the Kato[10] method, using 5 to 10 drops of blood drawn from the marginal vessels of the ear. This was mixed with dried oxalate (equivalent to 0.02 cc of 2% double oxalate solution). The E.S.R. in 2 guinea pigs serving as controls and injected intravenously for several days with 2 cc of 0.9% sodium chloride was never greater than 1%. In Table II a typical experiment is listed. It can be seen that the intravenous injection of hyaluronate leads to a definite increase in E.S.R. which usually reaches its maximum more than 1 hour after the injection. After the injections have been made daily for several days, the E.S.R. may remain elevated for 24 hours. Autopsy in some of the animals after repeated injections revealed no gross pathological changes.[†] The temperature of the injected animals showed no significant deviation from the normal.

The effect on E.S.R. of the intravenous injection of hyaluronidase in animals previously treated with hyaluronate was tested in 2 guinea pigs of 380 and 370 g respectively. In one guinea pig the hyaluronidase was injected immediately after hyaluronate. In the other, the enzyme was injected 4 hours later. In the former animal the E.S.R. remained 1%, while in the latter animal the E.S.R. rose from 0 to 27.5% but had returned to 0, 30 minutes after the injection of enzyme. The animals were apparently in perfect condition after the experiment.

In a similar experiment, the same 2 guinea pigs were injected several days later with 2.2 and 3.6 cc of 2% hyaluronate respectively. The E.S.R. in both guinea pigs before injection was <1 and rose to 12 and 16 respectively one hour after the hyaluronate injection. The second animal received one injection of hyaluronidase 65 minutes after the hyaluronate injection. Samples of blood were taken 10, 30, and 150 minutes after the injection of enzyme. The E.S.R. in these samples remained 1%. In the control animal, which received no enzyme, the E.S.R. 4 hours after the hyaluronate injection had risen to 45%.

Discussion. From literature data as well as from our experiments the conclusion can be drawn that a high degree of asymmetry of the molecules together with high particle weight are the causes of increased E.S.R. Among the known blood proteins fibrinogen, which since Fahraeus[2] has been thought largely responsible for increased E.S.R. in patho-

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[9] Morgan, W. T. J., and McClean, D., *J. Soc. Chem. and Ind.*, 1932, **51**, 912.

[10] Kato, K., *J. Lab. and Clin. Med.*, 1938, **23**, 980.

[†] We are greatly indebted to Drs. Beatrice Seegal and Emily Loeb, who did some of the preliminary experiments on guinea pigs in 1942.

TABLE II.
Sedimentation Rate in Guinea Pig Injected with Hyaluronate.*

Date	cc inj.	Sedimentation rates in % in 60 min.†		
		Before inj.	45 to 60 min. after inj.	3 to 4 hr. after inj.
6/19	0	2.5		
20	0	1		
21	1.8	0	4	
22	approx. 2		36	
23	1.9		21	
26	2.0	0	27	
27	approx. 1	10	27	47
28	2.0	5		51
29	2.0	27		17
30	1.9	0		54
7/ 1	1.8	54		58
5	1.0	1		46
6	1.8	20		61
7	1.0	9		62
8	1.9	21		
10	0	1		

* 2% solution of sodium hyaluronate in 0.9% sodium chloride.

† It should be noted that the pipettes used in the Kato method are calibrated in percent instead of in mm as in the Westergren method.

logical conditions, apparently fulfills this condition.[11]

The influence of the degree of aggregation is evidenced from the experiments with hyaluronate. Testis hyaluronidase at a pH of 7.4 leads only to depolymerization and not to hydrolysis as evidenced by the failure of opening of glucosidic linkages.[12]

It is apparent from the animal experiments that the intravenous injection of viscous hyaluronate leads to a considerable increase in E.S.R. similar to that induced by the addition *in vitro*. In contrast with the experiments of Youngner and Nungester[13] in which the E.S.R. returns to normal in about 40 minutes after the injection of type III pneumococcus polysaccharide, the increase produced by hyaluronate reaches a maximum in several hours, especially if injections are repeated. However we have not succeeded in maintaining a high E.S.R. in animals for prolonged periods. Presumably the substance is enzymatically depolymerized in the circulation.

Rouleaux formation is generally accepted as the immediate cause of increased E.S.R., although its precise mechanism is still unexplained. Fahraeus[2] argued against the presence of a "sticky" substance on the surface of the red cells and proposed a poorly defined colloidal phenomenon as the cause of the clumping. Experiments reported in this paper favor the older theory. Furthermore a highly viscous material of great asymmetry has been prepared from stromata. Apparently such highly asymmetrical substances in small concentrations exert a much greater influence on E.S.R. than do the known serum proteins. The occurrence of such substances in small concentrations is indicated by experiments on increased E.S.R. in various diseases, especially in rheumatic fever, where a highly purified testis extract leads to a specific decrease in E.S.R.

Summary. Addition of desoxyribonucleic acid, gelatin, fibrinogen, and especially hyaluronic acid to normal human citrated blood causes a considerable increase in E.S.R. Hyaluronic acid acts only in its highly polymerized form, thus suggesting that molecular weight is an important factor in this reaction, as well as

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[11] Cohn, E. J., *Trans. and Studies of College of Physicians of Phila.*, 1942, **10**, 149.

[12] Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

[13] Youngner, J. S., and Nungester, W. J., *J. Infect. Diseases*, 1944, **74**, 247.

asymmetry of the molecule. Hyaluronidase specifically reverses the action of hyaluronate on E.S.R. Intravenous injection of hyaluronate in guinea pig and rabbit leads to increased E.S.R. Intravenous injection of hyaluronidase promptly causes the increased rate to become normal.

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Effect of Wheat Germ on Creatinuria in Dermatomyositis and Progressive Muscular Dystrophy.*

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Milhorat, Weber, and Toscani[1] reported a decrease in the creatinuria of 2 patients with dermatomyositis who were given 125 g of fresh whole wheat germ daily. This effect was increased when the wheat germ was incubated *in vitro* with normal human gastric juice.

The present report presents some of the results of investigations carried out in an effort to identify the factor, or factors, in wheat germ having these effects. The subjects were 5 patients with dermatomyositis, and 3 patients with progressive muscular dystrophy. All subjects had extensive muscular wasting, and spontaneously excreted large amounts of creatine. The patients were given a creatine-creatinine-free diet and were maintained in a special metabolism ward where the diet and urinary collections could be rigorously supervised. After adequate periods of control the subjects were given various substances to test any possible effect on the creatinuria. Most of the substances were given daily to 4 or more of the subjects for periods of at least one week and usually for periods of 2 months. Briefly, the observations were as follows: only 3 of the patients with dermatomyositis (subjects A, B, and C), and one with progressive muscular dystrophy (subject D) were able to ingest as much as 125 g wheat germ daily. During the control periods the average daily levels of spontaneous creatinuria of the 3 patients with dermatomyositis were as follows: subject A, 0.450 g (maximum 0.524, minimum 0.405); subject B, 0.240 g (max. 0.275, min. 0.190); and subject C, 0.150 g (max. 0.240, min. 0.105). The average daily outputs of creatine were unchanged during the first 2 weeks of wheat germ administration, but during the third week they decreased to the following levels: subject A, 0.355 g (max. 0.406, min. 0.279); subject B, 0.082 g (max. 0.090, min. 0.078); and subject C, 0.080 g (max. 0.093, min. 0.072). Subject A was given wheat germ for a total period of 7 months. During the last 2 weeks of this period the average daily creatinuria was 0.310 g (max. 0.365, min. 0.256). Essentially similar results were observed in the patient with progressive muscular dystrophy (subject D). The average daily output of creatine of 0.350 g (max. 0.470, min. 0.338) gradually decreased during the third and fourth weeks of wheat germ feeding to levels of around 0.250 g (max. 0.266, min. 0.218). The excretion of creatine continued at these low levels for the next 2 weeks during which wheat germ was given, and for a period of 3 weeks after the administration had been stopped. The wheat germ given in this experiment had been vacuum packed at the mill and was kept in sealed cans until used. In a subsequent experiment wheat germ which had been stored and received in bulk was given to this patient in the same amounts as in the previous study. No effect on the creatine output was observed during

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* Aided by grants from the National Foundation for Infantile Paralysis, Inc., and the Nutrition Foundation, Inc.

[1] Milhorat, A. T., Weber, F. C., and Toscani, V., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 470.

the second period of feeding with this latter sample.

In all experiments the wheat germ was given in about 600 cc tomato juice daily, but neither 700 cc tomato juice nor 500 mg ascorbic acid daily affected the creatinuria. In 2 series of experiments in which wheat germ was incubated *in vitro* with normal human gastric juice at 37°C for 2 hours, 30 g of wheat germ daily had about the same effect as 125 g of the untreated material. Wheat germ incubated with brewers' yeast, extract of whole hog pancreas, or an extract of fresh beef liver had no more effect on the creatinuria than had fresh wheat germ.

Samples of wheat germ obtained from different sources varied considerably in their effects on creatinuria. Prolonged exposure of the wheat germ to air reduced the effects. Wheat germ defatted by continuous extraction with cold ethyl ether had definite effects, but these were less than those of the fresh product. These effects were not increased when the subjects received tocopherol by intramuscular injection. No effect on creatinuria was observed with wheat germ that had been defatted by extraction with ethylene dichloride. Water extracts of ether-defatted wheat germ were ineffective. The administration of large amounts of thiamin chloride, riboflavin, calcium pantothenate, para-amino benzoic acid, pyridoxine hydrochloride, nicotinic acid, nicotinamide, brewers' yeast, brewers' yeast concentrate, vitamin A, vitamin D, cottonseed oil (prime summer yellow grade), liver extract, soy bean phosphatides, bile salts, and hog pancreatin failed to affect the creatinuria. Wheat germ oil obtained by the cold-press method had doubtful effects when given in amounts of as much as 60 cc daily. A concentrate of tocopherols prepared by high vacuum molecular distillation from cold-pressed wheat germ oil was likewise ineffective.

The daily administration of 50 mg alpha-tocopherol by intramuscular injection for periods of as long as 6 months had no effect. The ingestion of yeast, ascorbic acid, pyridoxine, and other vitamin preparations during such periods did not affect the creatinuria. One patient with progressive muscular dystrophy (subject E) was given 20 g of soy bean lecithin daily. Aside from a slight increase there was no essential change in the urinary output of creatine during the first 10 days of administration. However, after the 15th day of lecithin ingestion the creatinuria dropped slowly to an average daily level of 0.150 g (max. 0.190, min. 0.110) from the previous control level of 0.270 g (max. 0.380, min. 0.240). When the administration of lecithin was discontinued the creatine output remained at the low level for about 6 days and then slowly rose to its previous level.

Summary. The administration of large amounts of fresh wheat germ which was vacuum packed and sealed reduced the creatinuria in 3 patients with dermatomyositis. Other samples of wheat germ, stored in bulk, were ineffective. In another subject with progressive muscular dystrophy the creatinuria was diminished in one experiment and unaffected during a second period of wheat germ feeding. Wheat germ incubated *in vitro* with normal human gastric juice had more effect on creatinuria than had the fresh product. Prolonged extraction with ethyl ether reduced but did not remove completely the factor or factors in wheat germ that lower the output of creatine. On the other hand, wheat germ defatted by extraction with ethylene dichloride was entirely without effect on the creatinuria. Of various vitamins and other substances investigated only soy bean lecithin reduced the creatinuria.

Acknowledgments are made for the following materials:

(a) Alpha-tocopherol in peanut oil solution for intramuscular injection and pyridoxine hydrochloride (Merck and Company, Inc., Rahway, N.J.)

(b) Soy bean lecithin and phosphatides (American Lecithin Company, Inc., Elmhurst, N.Y.).

(c) Wheat germ oil obtained by cold-press method (International Vitamin Corporation, New York, N.Y.).

(d) Ethylene dichloride-defatted wheat germ (Abbott Laboratories, North Chicago, Ill.).

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Influence of Thiamine Deficiency in *Macaca mulatta* on Susceptibility to Experimental Poliomyelitis.*

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For several years, we have been studying the possible influence of dietary deficiencies on the susceptibility of monkeys and mice to experimental poliomyelitis. The results with Swiss mice maintained on thiamine-, pantothenic acid-, and riboflavin-deficient diets have already been published.[1,2,3] Since little was known of the exact nutritional requirements of the rhesus monkey, preliminary studies with highly purified diets were necessary[4] before the experiments with the virus could be undertaken. The earlier thiamine studies[4] gave sufficient basis for approximate thiamine requirements of these animals; the more exact daily needs for this vitamin were determined individually for the 12 animals in Series 3.

Experimental. The methods used in the nutritional care of the animals have been described in a previous publication.[4] The basal thiamine-deficient diet consisted of the following parts per 100: sucrose 73, purified vitamin-free casein† 18, mineral salts 4, corn oil 2, and cod liver oil 3. Each animal received a daily vitamin supplement which included riboflavin 1 mg, nicotinic acid 5 mg, calcium pantothenate 3 mg, pyridoxine 1 mg, choline chloride 50 mg, *i*-inositol 100 mg, para-aminobenzoic acid 100 mg, and a bisulfite-treated liver extract (Wilson) solution equivalent to 2.5 g of original material. In Series 3, monkeys 131, 133, 134, and in Series 4, monkeys 148, 149, 151, 152, and 153 received in addition to the thiamine-deficient diet No. 1, an added pyruvate level, 20 μg of a commercial concentrate of biotin or of crystalline biotin (methyl ester), and "folic acid" equivalent to 5 g of liver fraction L.[5] The thiamine dosage for each animal was selected so that a gradual decline was produced.

The MV strain of poliomyelitis virus was used throughout. A 5% suspension of cord was centrifuged lightly, and the supernatant employed either by intracerebral injection in the first series, or by intranasal in the others. In Series 3 and 4, the first administration of the virus was preceded by irrigation of the nasal passages with a phosphate buffer at about pH 5.[6] Then, approximately 1 ml of the virus was dropped into each nostril on 3 successive occasions within 24 hours. The monkeys were observed at least twice daily for signs of poliomyelitis and/or acute polyneuritis; in some instances a careful differentiation between these conditions was essential. The signs of mild thiamine deficiency in the monkey were cessation of normal growth, anorexia, apathy, and an unkempt appearance; while those of frank polyneuritis were tremors, ptosis, muscular weakness, cachexia, and a temporary spastic paralysis of the limbs. These signs could be readily alleviated either by tube feeding or by intra-peritoneal inoculation with adequate quantities of the vitamin.

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* These studies were aided by a grant from the National Foundation for Infantile Paralysis, Incorporated.

[1] Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Infect. Dis.*, 1944, **74**, 41.

[2] Lichstein, H. C., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 3.

[3] Rasmussen, A. F., Jr., Waisman, H. A., and Lichstein, H. C., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 92.

[4] Waisman, H. A., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., *J. Nutrition*, 1943, **26**, 205; Waisman, H. A., and McCall, K. B., *Arch. Biochem.*, 1944, **4**, 265.

† SMACO.

[5] Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1943, **26**, 361.

[6] Schultz, E. W., and Gebhardt, L. P., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 728.

The signs of poliomyelitis were excitability, fine tremors, incoordination, ataxia, weakness of different muscle groups followed by flaccid paralysis; with this MV strain of virus, the cases commonly terminated with quadriplegia and death of the animal. The recorded time for the first signs of definite flaccid paralysis was calculated from the third intranasal insufflation. In all cases necropsies were performed on the dead or ether-sacrificed monkeys; histological studies were made in almost all instances. Whenever indicated, as in complicating infectious diseases, bacteriological studies were carried out to aid in the diagnosis.

In Series 1 (Table I), a small preliminary study, only 4 monkeys were employed, 2 of which were maintained on an optimum diet and 2 on a thiamine-deficient diet. All monkeys, regardless of diet, succumbed to typical clinical poliomyelitis and were sacrificed after quadriplegia.

A total of 7 thiamine-deficient monkeys were used in Series 2 (Table I). In order to maintain the animals, each one received 100-275 μg thiamine in one or two intraperitoneal injections at some time after inoculation of the virus. Although 4 of these animals never showed clinical signs of the infection, conclusions on this basis are difficult, because these monkeys were acutely deficient in thiamine, and died of polyneuritis within the average incubation period of the poliomyelitis infection. These 4 non-paralytic animals died 4, 7, 9, and 11 days, respectively, after the third intranasal insufflation of the virus suspension. The cord sections of 3 of these 4 monkeys, numbers 66, 69, 42, present lesions typical of poliomyelitis, while in the fourth monkey, No. 72, the lesions are less definitive, but are consistent with a diagnosis of early asymptomatic poliomyelitis.

The 3 remaining monkeys showed typical clinical signs of the infection and typical pathology. Because of the premature death of animals from the acute thiamine deficiency in this series, it was deemed advisable to establish the daily minimum thiamine requirements of each monkey more exactly, so that they might survive throughout the period necessary for establishing the virus disease.

In Series 3 (Table I), 18 monkeys were employed, 3 of which were control animals on a natural diet consisting of skimmed milk, corn, carrots, potatoes, and whole wheat bread. Fifteen were placed on a thiamine-deficient diet, but 3 of these were fed in addition, 4 g of sodium pyruvate daily by mouth, by stomach tube, or by intraperitoneal inoculation. Blood pyruvic acid levels were determined on all monkeys except the 3 on a natural diet.[4] In addition to determining the susceptibility of thiamine-deficient monkeys, this series was set up to test the hypothesis that the increased level of pyruvic acid resulting from thiamine deficiency might play a role in resistance to poliomyelitis. A typical protocol for one monkey, No. 126, in this series is given in Table II. Of the 12 deficient monkeys, 2, No. 58 and No. 127, showed no definitive paralysis, but No. 58 died one day after signs of weakness were observed. Histological studies of central nervous system sections from No. 58 showed definite although slight lesions of poliomyelitis, while No. 127, which never showed any signs of poliomyelitis, was sacrificed 28 days after virus was administered, and showed lesions suggesting abortive poliomyelitis of the type we designate "indeterminate." The other 10 deficient animals showed frank flaccid paralysis of 2 or more limbs.

The 3 control animals on a natural diet were in poor condition, and were used only because of the war and consequent shortage of animals. One of these monkeys, No. 102, died shortly after the first intranasal instillation of the virus, while the other 2, although weakness was recorded for both animals, never showed any signs of poliomyelitis. Monkey No. 101 died 11 days after virus was given, from acute dysentery, while No. 100 was sacrificed 56 days following the start of the experiment. Microscopic study of central nervous system sections from these 2 animals revealed "indeterminate" lesions not definitely those of poliomyelitis but consistent with the suggestion of early damage by that virus. Since the monkeys maintained on a thiamine-deficient diet plus pyruvate were from the same shipment as the control animals, the cause of the resistance of 2 of these animals, No. 133 and No. 134, to poliomyelitis may be queried. It may have

TABLE I.
Influence of Thiamine Level and of Increased Pyruvate on Susceptibility of *M. mulatta* to MV Strain Poliomyelitis Virus.

Series No.	Monkey No. Sex	Wt, kg	Diet No.*	First signs of paralysis (days)	Pathology†	Remarks
I	36 F	2.3	1	8	3+	Quadriplegia
	37 F	2.4	1	10	No sections	"
	38 F	2.1	2	7	" "	"
	39 F	2.3	2	8	" "	"
II	40 M	2.7	1	8	4+	Quadriplegia; polyneuritis
	42 M	2.9	1		2+ cervical 1+ lumbar	Polyneuritis; died 7 days following inoc.; no paralysis
	51 M	2.8	1	7	No sections	Quadriplegia; polyneuritis
	66 M	3.3	1		1+	Polyneuritis; died 4 days following inoc.; no paralysis
	69 F	2.0	1		3+	Polyneuritis; died 9 days following inoc.; no paralysis
	70 F	2.7	1	7	4+	Polyneuritis; flaccid par. of one limb
	72 F	1.5	1		±	Polyneuritis; died 11 days following inoc.; no paralysis
III	27 M	4.8	1	9	3+	Polyneuritis; flaccid par. of 2 limbs
	28 F	3.6	1	6	4+	Quadriplegia
	33 F	4.3	1	18	4+	"
	52 F	2.7	1	9	3+	Flaccid par. one limb
	55 M	3.4	1	6	3+	Polyneuritis; quadriplegia
	58 M	3.6	1		1+	No definite par.; marked ataxia, tremors, weakness 8th day following inoc.
	75 M	3.3	1	7	3+	Polyneuritis; flaccid par. of 2 limbs
	93 F	2.8	1	12	4+	Quadriplegia
	107 M	2.4	1	13	1+	Hemiplegia
	108 M	2.9	1	6	3+	Quadriplegia
	126 F	2.2	1	12	4+	Quadriplegia; polyneuritis
	127 M	2.0	1		±	Sacrificed 28 days following inoc.; no signs
	100		3		±	Sacrificed 56 days following inoc.; irritable, weak 7 days
	101		3		±	General weakness, especially legs, 10 days following inoc.; died 11th day; clinical and bact. evidence of dysentery
	102		3			Died after first intranas. insufflation; dysentery
	131 M	2.4	4	7	4+	Quadriplegia
	133 F	2.0	4		±	Sacrificed 33 days following inoc.; no signs
	134 M	2.1	4		±	Sacrificed 29 days following inoc.; no signs
IV	16 F	1.9	2	10	4+	Quadriplegia
	144 M	2.7	2	7	4+	"
	146 F	4.0	2	9	4+	"
	147	4.1	2	9	4+	"
	25 F	2.0	2	7	3+	"
	145	4.6	2		±	Sacrificed 33 days following inoc.; no signs
	148	2.5	4	8	4+	Quadriplegia
	149 F	2.1	4	9	3+	Flaccid par. of 2 limbs
	151 M	2.5	4		±	General weakness, no signs of paralysis; died 22 days after inoc.
	152 M	3.4	4	8	3+	Flaccid par. of 2 limbs
	153 M	3.0	4	8	4+	Quadriplegia

* 1. Thiamine deficient diet; 2. Optimum diet (control animals); 3. Natural diet (control animals); 4. Modified thiamine-deficient diet plus 4 g sodium pyruvate per day.

† Stages of poliomyelitis lesions in *Macaca mulatta* as used in Table I:

± Indeterminate: A few (4 to 6 or 8 per section) focal areas of microglial infiltration; most of the ganglion cells appear normal; some, however, show chromatolysis with margination of Nissl substance and a few show more marked degeneration with satellitosis of microglial cells and lymphocytes; congestion of vessels with occasional slight hemorrhage. Only an occasional polymorphonuclear leucocyte observed in the sections; no meningitis; no perivascular round cell infiltration.

This picture is considered consistent with early asymptomatic poliomyelitis but is not pathognomonic; in this instance, since poliomyelitis virus was introduced, these lesions are probably due to that virus.[7]

1+ Slight perivascular round cell infiltration especially in medulla; larger amount of microglial infiltration than in the "indeterminate" category; extensive chromatolysis with margination of Nissl substance and eccentric nuclei; necrosis of few ganglion cells with invading microglial phagocytes and lymphocytes but very few polymorphonuclear leucocytes; usually slight hemorrhage.

Animals with this degree of damage have commonly shown tremors, ataxia, and weakness, but no paralysis.

2+ Typical lesions with perivascular cuffing, oedema, easily recognized necrosis of nerve cells with neuronophagia, composed of about equal numbers of microglial cells, lymphocytes, and polymorphonuclear leucocytes. Disappearance of an appreciable number of ganglion cells.

3+ Marked typical lesions with extensive nerve cell necrosis and neurono-phagocytosis; most of the invading cells involved in this process are polymorphonuclear leucocytes. Round cell meningitis.

4+ Overwhelming typical lesions with hardly a normal ganglion cell left at any level of the cord studied. Many polymorphonuclear leucocytes throughout.

TABLE II.
Typical protocol of a *M. mulatta* Monkey Maintained on a Thiamine-deficient Diet and Given MV Virus by the Intranasal Route (Monkey No. 126).

10/ 1/43	M-3 + 2½ g sulfited liver extr. per day.		
10/15	First signs of thiamine deficiency. Minimum thiamine requirement established as 50 μg per day. Blood pyruvic acid determination after 36 hr starvation = 3.4 mg per 100 ml blood.		
11/24	MV virus given intranas. 3 times during previous 24 hours. Temp. 103°F.		
11/29	Blood pyruvic acid level = 3.02 mg per 100 ml blood.		
11/30	Temp. 103.3°F.		
12 /1	"	104.0	very irritable.
12/ 2	"	104.9	continued irritability.
12 /3	"	104.9	" "
12/ 4	"	104.2	" "
12/ 5	"	106.2	face hyperemic; marked irritability; slight incoordination of movements.
12/ 6	"	104.2	fine head tremors; flaccid paralysis of both arms.
12/ 7	"	100.2	ptosis; quadriplegia; marked head tremors
12/ 8	"	97.4	no significant change; respiration quite normal.
12/ 9	Found dead 8 a.m. Necropsy performed at 9 a.m. No gross changes observed. *Micropathology:* 4+. Extremely acute poliomyelitis; not a single normal nerve cell observed in the cord sections. Parenchymatous degeneration of all the viscera; marked hyperplasia of germinal centers of spleen. *Conclusion:* Marked acute poliomyelitis.		

been due in part to the generally poor nutrition of the animals.

Series 4 (Table I) presents another attempt to determine whether with monkeys free from dysentery and with no complicating deficiencies, the pyruvate hypothesis has any validity. One of 5 monkeys, No. 151, fed a thiamine-deficient diet plus added pyruvate showed no sign of poliomyelitis infection but died 22 days after inoculation with interstitial pneumonia. One of the controls, No. 145, maintained on a complete diet also showed no signs of the disease and was sacrificed in 33 days. Both of these animals showed the "indeterminate" pathology as described in Table I.

Summary. The influence of thiamine deficiency on the susceptibility of *M. mulatta* to experimental poliomyelitis has been studied in 40 animals. The results indicate that this species, when deficient in thiamine, does not exhibit an increased resistance to poliomyelitis virus in contrast to the well-demonstrated resistance of Swiss mice similarly fed.[1,8]

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[8] Foster, C., Jones, J. H., Henle, W., and Dorfman, *J. Exp. Med.*, 1944, **79**, 221; 1944, **80**, 257.

14834

Dietary Granulocytopenia in Rats Corrected by Crystalline *L. casei* Factor ("Folic Acid").

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Granulocytopenia and anemia are produced in rats by the administration of sulfonamides in purified diets.[1,2] These blood dyscrasias are corrected by liver concentrates containing "folic acid"[1,2,3] and by crystalline *L. casei* factor or vitamin B_c.[4] In the present studies granulocytopenia has been observed in a small percentage of rats fed a purified diet without sulfonamides. Crystalline *L. casei* factor was effective in the correction of this blood dyscrasia. The occurrence of granulocytopenia in rats fed stock rations has not been observed in this laboratory or reported by others.[5,6]

Methods. Albino rats of Wistar and Osborne and Mendel strains were fed an experimental diet at weaning. 171 rats were fed purified diet No. 956.* 14 rats were fed another purified diet No. 937.† 21 rats were fed a sulfasuxidine-containing purified diet No. 945. It consisted of essentially the same ingredients as diet No. 956 but contained sulfasuxidine as 1% of the diet in place of 1% of sucrose.

The technic for making total leucocyte and polymorphonuclear cell counts and hematocrit determinations was the same as in previous studies.[7] With rats fed purified diets No. 956 and No. 937, blood counts were made 30 to 50 days after starting the diet. At this time, 155 of these 185 rats were used in another experiment and they are not considered further in this study. On the other 30 rats, blood counts were repeated at irregular intervals. With rats fed the sulfasuxidine-containing diet No. 945, a single count was made 36 days after starting the diet.

Treatment was by the oral administration of 25 γ of crystalline *L. casei* factor[8]‡ for each of 4 days. Before starting treatment to correct granulocytopenia, 1 to 4 repeat counts were made over a period of 1 to 4 weeks. In every case, counts were made on the day when treat-

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[1] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Asburn, L. L., *Pub. Health Rep.*, 1942, **57**, 1559.

[2] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, **98**, 20.

[3] Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F., *J. Biol. Chem.*, 1943, **148**, 721.

[4] Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1943, **58**, 1542.

[5] Scarborough, R. A., *Yale J. Biol. and Med.*, 1931, **3**, 267.

[6] Creskoff, A. J., Fitz-Hugh, T., Jr., and Farris, E. J., in *The Rat in Laboratory Investigation*, edited by J. G. Griffith and E. J. Farris, Philadelphia, J. B. Lippincott Co., 1942, p. 351.

* Diet No. 956 consisted of sucrose 67.88 g, casein (Labco) 18.0 g, Crisco 8.0 g, and salt mixture No. 550 (1) 4.0 g. Into this diet was incorporated 2 g of corn oil containing 16,000 units of vitamin A and 1,600 units of vitamin D (Natola), 0.12 g of ethyl laurate containing 12 mg of α-tocopherol, 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 1 mg of pyridoxine hydrochloride, 4.0 mg of calcium pantothenate, 2 mg of niacin, 200 mg of choline chloride, .001 mg of biotin, and 0.4 mg of 2-methyl-1,4-naphthoquinone.

† Diet No. 937 consisted of anhydrous dextrose 71.76%, casein (Labco) 18%, cod liver oil 2%, cottonseed (Wesson) oil 3%, ferric citrate (iron 18.09%) 1.16%, copper sulfate ($5H_2O$) .08%, and Salt mixture No. 550 4%. Each rat received a daily supplement of 100 γ of thiamine hydrochloride, 200 γ of riboflavin, 100 γ of pyridoxine hydrochloride, 200 γ of calcium pantothenate, 1 mg of niacin, 10 mg of choline chloride, 0.5 γ of biotin (Merck), and 20 γ of 2-methyl-1,4-naphthoquinone.

[7] Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Phys.*, 1944, **142**, 604.

[8] Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

‡ The crystalline material used in the present studies was furnished through the courtesy of E. L. R. Stokstad, B. L. Hutchings, and N. H. Slobodkin of Lederle Laboratories.

ment was started and on the day following the last treatment.

The term "granulocytopenia" is used in this report to indicate a total polymorphonuclear cell count of less than 500 cells per cu mm.

Results. Granulocytopenia was observed in 6 of 185 rats fed purified diets for 30 to 50 days. Thirty rats were continued on these diets (155 were used in another experiment). Two of the 30 rats developed granulocytopenia after 125 days. Average values for the entire group of 185 rats 30 to 50 days after starting the diet were as follows: polymorphonuclear leucocytes: 2,448 cells per cu mm; total leucocytes: 13,931 cells per cu mm; hematocrit: 41.5 vol.%. In 2 rats with granulocytopenia there was a very slight and gradual increase in cell count without treatment; 1 rat died before treatment was started and 5 rats in which granulocytopenia was persistent were treated. Crystalline *L. casei* factor administered to these 5 rats with persistent granulocytopenia resulted in a rapid and striking response in every case (Table I). A concomitant, though lesser increase in non-polymorphonuclear leucocytes was noted. A relapse to granulocytopenic levels approximately one month following the responses to treatment was found in 3 of the 5 rats. Two of these rats were treated again and responded as well as after the initial treatment. A second relapse occurred a month later in one of these rats. Weight loss or poor growth, and sluggish activity were noted in all the rats prior to treatment. Weight gains and a general clinical improvement were noted in some of the rats following treatment. An hematocrit value under 30 vol.% was found in only one of this series of 185 rats.

Twenty of 21 rats fed the sulfasuxidine-containing purified diet No. 945 for 36 days had granulocytopenia. Only 1 rat had an hematocrit value under 30 vol.%.

Discussion. These data indicate that granulocytopenia, correctable by the *L. casei* factor, may occur in rats solely as the result of the ingestion of a highly purified diet. It is suggested that factors which may contribute to the infrequent appearance of this condition are the presence of body stores of the *L. casei* factor at weaning, contamination of the diet

TABLE I. Production of Dietary Granulocytopenia and Correction by Crystalline *L. casei* Factor ("Folic Acid.").

			Values before treatment				Values after treatment*			
Rat No.	Diet	No. of days on diet	Polymorphonuclear cells per cu mm	Total leucocytes per cu mm	Hematocrit vol. %	Wt gain in preceding 10 days, g	Polymorphonuclear cells per cu mm	Total leucocytes per cu mm	Hematocrit vol. %	Wt gain in 10 days, g
1	937	134	100	12,350	40	—15	5,300	27 750	42	0
		169†	50	4,750	38	— 3	16,600	34,550	43	—11
		203†	250	650			‡			
2	937	135	0	2,100	27	—39	3,500	9,000	27	—10
		167†	350	2,300	36		‡			
3	956	64	100	5,500	37	+11	2,800	10,300	39	+37
		126†	450	3,950	40	— 2	2,700	8,750	42	+29
4	956	43	450	3,150	43	—10	3,900	11,650	35	+17
5	956	33	450	2,700	36	+ 5	3,050	10,150	41	+24
6	937	30	200	6,350	40		‡			
7	956	30	400	7,050	41		§			
8	956	30	350	6,150	40	+ 5	‖			

* Treatment was by the oral administration of 25 γ of crystalline *L. casei* factor ("folic acid") for each of 4 days. Counts were made immediately before treatment and on the day following the last treatment. † Note relapse to granulocytopenic level. ‡ Died before treatment was started.

§ No treatment given. Values after 36 days on diet were 800 polymorphonuclear cells and 7,350 leucocytes per cu. mm.

‖ No treatment given. Values after 41 days on diet were 850 polymorphonuclear cells and 6,700 leucocytes per cu. mm.

and the low requirement of the rat for this vitamin. It also appears plausible from analogous studies on vitamin K deficiency[9–12] that intestinal synthesis of the *L. casei* factor, known to occur,[13] may be a significant factor.

It should be pointed out that the number of cases of granulocytopenia noted in this series of rats does not necessarily represent the maximal incidence. Observations were not made over a long period of time in most rats, and the purified diets used are not considered to be completely free from "folic acid."

The absence of severe anemias in this series of rats does not prove that the erythropoietic system was unaffected. It has been shown that when sulfasuxidine-fed rats with normal hematocrit values are subjected to repeated bleedings, red blood cell regeneration may be very inadequate.[7] Similar studies have not been carried out as yet in a large series of rats fed purified diets without sulfonamides.

Summary. 1. Granulocytopenia was observed in a small percentage of rats fed purified diets without sulfonamides. 2 Crystalline *L. casei* factor ("folic acid") administered to 5 rats with granulocytopenia corrected the blood dyscrasia in every case. One or more relapses occurred in 3 of the rats. Crystalline *L. casei* factor ("folic acid") corrected the granulocytopenia in relapse.

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[9] Dam, H., and Glavind, J., *Z. f. Vitaminforschung*, 1939, **9**, 71.

[10] Greaves, J. D., *Am. J. Phys.*, 1939, **125**, 429.

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[12] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1944, **59**, 832.

[13] Mitchell, H. K., and Isbell, E. R., *The University of Texas Publication*, No. 4237, 1942, 125.

14835

A Heretofore Undescribed Phase-Variation in Salmonella.

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Andrews[1] discovered the phenomenon of *phase-variation* in bi-phasic Salmonella types. If a motile strain f.i. *S. typhi murium* (IV.V;i-1,2,3 . . .) is plated out on a moist agar plate, single colonies after 24 hours present either the i or the 1,2,3 phase. On further subculture either phase splits off the other phase in a certain percentage, generally not exceeding 5%. The graphic expression of this phase-variation reads: IV.V; i ⟷ 1,2,3.

The two phases present in the two kinds of colonies are easily identified by known monophasic antisera or by antibody production. If a hitherto unknown phase appears and is not agglutinated by known antisera, it can be classified by immunization of rabbits with single colonies of this phase.

The unusual phase-variation which we are reporting differs from this pattern. A culture isolated from the blood stream of a severely sick elderly woman* showed culturally and serologically almost all the characteristics of *S. typhi murium*. The somatic antigen was IV.V, the flagellar antigen bi-phasic, one phase representing the 1,2,3 antigen. The other phase, however, was not the presumed i nor was it agglutinable by any one of our complete set of H-antisera. On occasion a trace of a 1,2,3 reaction was seen, suggesting that this phase, for simplification called X phase, may split off the 1,2,3 phase. Plated out from single colonies the X phase split off 1,2,3. The original 1,2,3 phase on the other hand split off

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[1] Andrews, F. W., *J. Path. and Bact.*, 1922, **25**, 505.

* The patient died. Culture and clinical data were kindly submitted by Dr. Kantrowicz, Beth Moses Hospital, New York.

TABLE I.
Effect of Gard's Technic on the H-Antigens.

Phase	Serum applied	Direct result	Consecutive phase variation
1,2,3	1,2,3-antiserum	X phase	X ⟷ 1,2,3
1,2,3	i- ”	1,2,3 ”	1,2,3 ⟷ X
X	1,2,3- ”	i ”	i ↗ ↖ ↙ ↘ 1,2,3 ⟷ X
X	i- ”	1,2,3 ”	1,2,3 ⟷ X
X	i-antiserum + 1,2,3-antiserum	immobilized X phase	X ⟷ 1,2,3

the X phase, both in a ratio of 25%. Thus, a phase-variation was observed, the X phase, however, remained inagglutinable also when derived from the 1,2,3. Both phases were motile, the X phase to a lesser degree. It was, however, not difficult to obtain well motile inagglutinable X phases in broth culture. Six-hour growth in broth, formolized, served as antigen for the immunization of rabbits. The resulting antiserum contained O-antibodies against IV.V and H-antigens. The latter were not of a new type; instead they represented a mixture of antibodies against the 1,2,3 and the heretofore missing i phase in almost equal amounts. Titers were: for O 1:1280, for i 1:6400; for 1,2,3 1:6400. The antigen of the X phase was agglutinated by this serum in granular form up to the serum's O titer 1:1280; no floccular (H) agglutination was observed. Absorption of the serum by normal *S. typhi murium* culture removed all antibodies and did not leave behind any substances reacting with the X phase. This phase, therefore, must contain the 2 typical H-antigens in almost equal amounts. That in itself is a rare incident, having been only once previously described in a culture of *S. paratyphi B* by Kauffmann.[2] But under the observed conditions our X phase cannot be agglutinated although it readily produces antibodies. In order to ascertain whether inhibiting substances were covering the H-antigens similar to the Vi in somatic antigens, recourse was had to sonic vibration.[†] No liberation of H-phases could be detected. Electron microphotography performed through the courtesy of Dr. G. Shwartzman of Mt. Sinai Hospital, N.Y., did not reveal any morphological differences between the X phase and the normal *typhi murium* culture.

As mentioned before the X phase splits off the 1,2,3 phase readily; spontaneous development of an i phase could not be obtained under the usual or changed growth conditions. Neither growing at different temperatures nor change of media—glucose broth, brain-heart infusion broth, ascitic broth, etc.—nor the shift of pH to the acid or alkaline side had any effect. Daily transplants were performed for 2 months and prolonged cultivation in the same broth was used without success. Ultraviolet radiation and repeated freezing and thawing were as unsuccessful as enteral or parenteral passage through mice. Successive transfers from mouse peritoneum to mouse peritoneum in 20 generations did not affect the X phase, not even a 1,2,3 phase developed in this prolonged experiment.

To summarize: Both the original phases (X and 1,2,3) spontaneously split into 1,2,3 and X individuals. In no instance, under greatly varied conditions, did an i phase develop.

Gard's technic of evoking the appearance of suppressed phases by addition of antiserum of the other phase was then applied to the two phases. The result of these experiments may be seen in Table I.

The effect of the Gard treatment on the X phase is twofold. Under the application of an anti-i serum a 1,2,3 phase is liberated, confirming the actual presence of this phase previously found in spontaneous phase variation. Addition of an anti-1,2,3 serum, however, brought out the hitherto missed i phase. Combined treatment with i and 1,2,3 antiserum immobilizes the culture without appearance of any other phase. On subculture the "induced" i phase reproduces 3 phases, the i, X, and 1,2,3, the latter 2 forming about 5% of the colonies. The action of 1,2,3 serum on the original 1,2,3 phase sets the X phase free, the i antiserum has no influence.

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[2] Kauffmann, F., *Zbl. Bakt.*, 1934, **132**, 20.

[†] The authors are indebted to Dr. David Green and Mr. P. K. Stumpf of Columbia University, College of Physicians and Surgeons, Department of Medicine, who kindly performed the sonic vibration.

Altogether, 3 well defined phases were recovered by this method: X, i, and 1,2,3. Later in animal experiments a fourth variant was found: a well agglutinable i + 1,2,3 phase in single colonies, comparable to that found by Kauffmann in *S. paratyphi B* (b + 1.2).

Living cultures of the original strain and of the various phases were unusually virulent for animals. Of 5 rabbits, one guinea pig, and more than 100 mice which were fed or injected, only one mouse survived. Among the fatalities was one rabbit which had been immunized previously with a formolized culture. The minimal fatal doses used consisted of 10^{-8} dilutions of broth, representing about 50 organisms. Computed on the average time of survival, the X phase exerted the highest pathogenicity in mice, the 1,2,3 phase the lowest. The number of animals involved in the study of each phase was not large enough to allow statistical evaluation of results. When mice were fed only the X phase, both X and 1,2,3 colonies were recovered from blood and stool. When the 1,2,3 phase was fed, again both X and 1,2,3 colonies were isolated from blood and stool. Of the six mice fed the "induced" i phase, five died, one survived, and the cultures yielded i, 1,2,3, and X colonies from the blood and feces. From the one surviving mouse pellets of feces were taken after 19, 41, 70, and 103 days. The first specimen showed only i colonies, the specimen secured on the 41st day yielded i colonies and colonies which surprisingly turned out to be bi-phasic, i + 1,2,3. The specimen collected on the 70th day displayed i, 1,2,3, and i + 1,2,3 colonies, and the one collected on the 103d day yielded i and i + 1,2,3 colonies. In animals and on subculture the i + 1,2,3 phase yielded the i, 1,2,3, and i + 1,2,3 phases but never the X phase. This i + 1,2,3 phase seen in about 10% of the single colonies, might actually be identical with the X phase which now became agglutinable. A formolized 6-hour broth culture of this variant was used for rabbit immunization. The resulting antiserum was active against the O (IV.V), i and 1,2,3 antigens. The fact that after removal of the O-antibody by specific absorption the remaining H-antibodies did not agglutinate the X phase indicates there is no difference between this serum and that produced against the X phase—the X phase itself has retained its inagglutinability now for 11 months.

Summary and Conclusions. The natural appearance of an inagglutinable but antigen-producing "mixed" phase of a *S. typhi murium* is described. The phase i, the presence of which could not be elicited in the original cultures, was brought out by the use of Gard technic. An agglutinable "mixed" phase was produced *in vivo*. Data on the different phase variations of this Salmonella variant are given.

The described observations raise a number of theoretical questions unanswerable for the time being. This paper, therefore, limits itself to a statement of the facts.

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A New Salmonella Type: *S. Virginia.*

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Culture 2255 was received on October 10, 1944, from A. Corpening, Director of Laboratories, Department of Health, Richmond, Va., where it had been isolated by F. Spindle from the stool of a 40-year-old woman. The patient had suffered from 4 episodes of nausea, diarrhea, and fever, each lasting 12-72 hours, in the period from June until October, 1944. Her blood serum, received through the courtesy of Dr. J. P. Lynch, St. Luke's Hospital, Richmond, Va., on October 23 and November 7, 1944, agglutinated the culture in a dilution 1:80.

The isolated organism is a gram-negative motile rod possessing the biochemical characteristics of the genus Salmonella. Dextrose, maltose, mannite are fermented with gas formation. Lactose, sucrose, salicine, are not attacked. Sorbitol, dulcitol, xylose, rhamnose, arabinose are promptly acidified. Inositol and *d*-tartrate show transitory acidification with subsequent reversion to alkalinity after 24 hours. H_2S is produced, no indol; gelatin is not liquefied.

On feeding, the culture is not pathogenic for mice, but it was still being excreted 35 days after feeding. Of 4 mice injected s.c. with 0.1 cc of a 20-hour broth culture, 2 died after one day, one after 3 days, and one survived. The organism was recovered from the heart blood of the dead animals.

The O-antigen. Boiled bacilli were agglutinated only by sera derived from *S. newport* (VI.VIII), *S. kentucky* (VIII.XX), and *S. amherstiana* (VIII); by the latter to its end titer. The presence of an VIII antigen is thus evidenced. This antigen in combination with the VI is found in the VI.VIII group (12 types are known by now) and associated with the XX antigen in *S. kentucky*. Only *S. amherstiana* (VIII;eh-1.7) recently described by Edwards and Bruner[1] displays the VIII antigen with no additional factors. Single factor antisera against VI or XX did not agglutinate the new strain, nor did sera containing VI antibodies (*SS thompson* (VI.VII), *carrau* (VI.XIV.XXIV), *onderstepoort* (I.VI.XIV. XXV)). In absorption tests the culture removed completely the VIII fraction from VI.VIII, VIII.XX and VIII sera leaving VI and XX antibodies untouched. This speaks for the presence of VIII either as the only antigenic factor (like in *S. amherstiana*)

TABLE I.

Antigen		Serum									
		S. newport		*S. kentucky*		*S. amherstiana*		*S. virginia*			
									Absorbed with		
		Unabsorbed	Abs. with *virginia*	Unabsorbed	Abs. with *virginia*	Unabsorbed	Abs. with *virginia*	Unabsorbed	*newport*	*kentucky*	*amherstiana*
S. georgia	VI.VII	80	80	0		0		0			
S. newport	VI.VIII	**640**	80-160	160	0	800	0	2560	0	0	0
S. kentucky	VIII.XX	160	0	**320**	160	800	0	2560	0	0	0
S. amherstiana	VIII	80	0	160-320	0	**1600**	0	2560	0	0	0
S. virginia		160	0	160	0	8-1600	0	**2560**	0	0	0

Figures give highest agglutinating dilution.
0 = negative 1:40.

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[1] Edwards, P. R., and Bruner, D. W., *J. Immunol.*, 1942, **44**, 319.

or in a new combination VIII . . .

An O-antiserum prepared with boiled culture material agglutinated the homologous strain and *SS. newport, kentucky, amherstiana* almost uniformly to its end titer, but did not attack types with the VI.VII, VI.XIV.XXIV, and I.VI.XIV.XXV antigens. VIII containing types (*SS. newport, kentucky, amherstiana*) removed all antibodies for themselves and for the new strain from the serum. Thus, the exclusive presence of the VIII antigen and its identity with that of the other types could be established. The newly found VIII seems even more complete than that of *S. amherstiana* which in Edwards and Bruner's experience did not fully cover the VIII of the *newport* type.

The H-antigen. The strain's flagellar antigen is a monophasic *d* as is present in *S. typhi* and *S. wichita*. Testing of numerous single colonies did not reveal another phase, nor did the culturing in semi-solid agar with addition of *d* antiserum (Gard's technic). Living and formolized antigens are readily agglutinated by sera prepared with types endowed with the *d* antigen (*SS. stanley, amersfoort, muenchen, typhi, shangani, niloese, wichita*). The sera agglutinate the strain 2255 to approximately 50% of their relative titers. On the other hand, this strain removes about 75% of their agglutinins by absorption. A serum prepared with formolized broth culture of the strain agglutinates all *d* containing types to high titers, some of them to the end titer. Slight differences observed are in agreement with previous statements by Kauffmann,[2] Hormaeche and Peluffo,[3] and Edwards and Bruner.[4] These authors found differences in the *d* of different types and emphasized the complexity of that flagellar antigen.

The strain 2255 represents a new pathogenic Salmonella type. It has been named *S. virginia* after its place of isolation.

Summary. *S. virginia*, a new Salmonella type, has been described. It was isolated from a case of human enteritis and has the formula VIII; d —.

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[2] Kauffmann, F., *Z. Hyg.*, 1937, **120**, 177.

[3] Hormaeche, E., and Peluffo, C. A., *Arch. Urug. de Med. Cir y Esp.*, 1941, **14**, 217.

[4] Edwards, P. R., and Bruner, D. W., *Am. J. Hyg.*, 1942, **34**, 121.

14837

Cystine Inhibition of the Succinoxidase System. III. Effect of Dialysis.*

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During the course of a previous investigation on the inhibition of the succinoxidase system by cysteine and cystine, we showed that the concentration of four-carbon dicarboxylic (C-4) acids in the tissue homogenate was a determining factor.[1] Subsequently, we showed that with either cysteine or cystine added to the reaction mixture (cytochrome *c* present), the inhibition was entirely due to cystine.[2]

All inhibitors acting on the sulfhydryl group in succinic dehydrogenase will be affected by the presence of any C-4 acids or related substances. Since considerable importance has been attributed to the action of such inhibitors in determining whether or not enzymes function by means of sulfhydryl groups,[3,4] some means must be found to eliminate these interfering substances from the homogenate before quantitatively accurate data can be obtained.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided by a grant from the Rockefeller Foundation.

[1] Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, 1944, **5**, 191.

[2] Ames, S. R., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 109.

[3] Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, 1943, **26**, 391.

[4] Barron, E. S. G., and Singer, T. P., *Science*, 1943, **97**, 356.

It is the purpose of this communication to discuss an attempt to remove such interfering substances by means of dialysis. While much extraneous material and a considerable portion of the C-4 acids present were removed, an inhibition approaching 100% could not be secured under the experimental conditions.

White mice of an inbred Swiss strain were used and, after weaning, were maintained on stock ration[†] and water *ad libitum* plus occasional greens. Adult male white rats of the Sprague-Dawley strain were also used and were maintained on the same ration as the mice.

Tissue homogenates were prepared by the technic of Potter and Elvehjem[5] modified as described previously.[1,2] The activity of succinic dehydrogenase was determined by the method of Potter[6] as modified by Potter and Schneider.[7]

In order to secure reproducible results the *incubation time, i.e.,* the time the homogenate was incubated at 37°C before the inhibitor was added, and the *contact time, i.e.,* the time the inhibitor was in contact with the enzyme before the substrate was added, were carefully controlled. The numerical inhibitions recorded are valid only for a similar preparation under identical conditions.

A method of dialysis was selected in which the sample was constantly in motion.[‡] By this means dialysis was facilitated, thus shortening the time of dialysis and subsequent losses in activity. Regenerated cellulose tubing[§] was used as the dialysis membrane. Redistilled water was used and was frequently changed. The temperatures were roughly maintained as indicated by means of an external water or ice bath. The dry weights of the samples were determined by evaporating to constant weight in open crucibles in an electric oven at 110°C.

The cystine was recrystallized by the method previously described.[2] A stock solution of 0.72 mg per ml in 0.05 M sodium phosphate buffer was prepared by heating to boiling and this solution remained supersaturated for 12 hours or more at room temperature.

The temperature at which the homogenate was dialyzed proved to be an important factor in determining activity losses. A comparison of Curves I and II of Fig. 1 shows that the lower the temperature the less the loss in activity. At 25°C no activity remained after 6 hours of dialysis of a 2% homogenate, whereas at 0°C a similar preparation exhibited 64% of its original activity after 6 hours dialysis. Similar results were obtained on storage of homogenates *in vitro.* Twelve

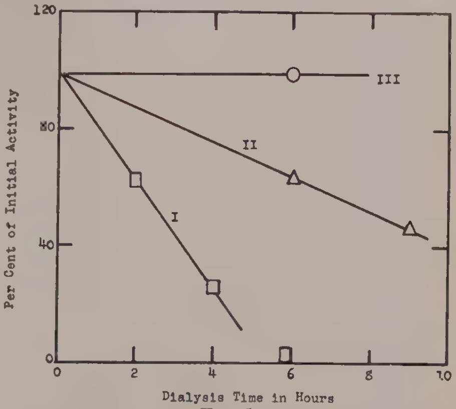

FIG. 1.

Effect of dialysis on the total succinoxidase activity. Assay performed as described in text. Incubation time of 40 minutes and contact time of 30 minutes with no inhibitor added.

Curve I—2% homogenate of mouse kidney dialyzed at 25°C.

Curve II—2% homogenate of mouse kidney dialyzed at 0°C.

Curve III—10% homogenate of mouse kidney dialyzed at 0°C.

[†] B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, New York.

[5] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[6] Potter, V. R., *J. Biol. Chem.*, 1941, **141**, 775.

[7] Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, 1942, **142**, 543.

[‡] A weighted dialysis bag containing the sample was suspended in water from a rubber band which was stretched from a fixed support to an eccentric rod rotating at about 100 r.p.m. This stirring rod was so bent that the rubber band was stretched about 4 cm with each revolution. By this means the suspended dialysis bag was alternately raised and lowered, this resulting in a "milking" effect which compensated in part for the normal increase in volume of the dialysate.

[§] Visking cellulose sausage casings, The Visking Corporation, Chicago, Illinois.

TABLE I.
Dialysis and Storage of Tissue Homogenates.

Exp.	Description	% inhibition Before treatment	% inhibition After treatment	% increase in inhibition	Time in hrs	% increase in inhibition per hr
1	Mouse kidney—2% homogenate dialyzed at 25°C	41	56	37	4	9.2
2	Mouse kidney—2% homogenate dialyzed at 0°C	42	57	36	6	6.0
3	Mouse kidney—10% homogenate dialyzed at 0°C	42	55	31	7½	4.1
4	Mouse kidney—10% homogenate stored at 0°C	42	45	7	7½	0.9
5	Mouse kidney—10% homogenate stored at 0°C with the addition of cytochrome *c* to 10^{-4} M and coenzyme I to 75 γ/ml	40	53	32	3½	9.3
6	Rat liver—10% homogenate dialyzed at 0°C	19	28	47	5½	8.6
7	Rat liver—10% homogenate stored at 0°C	19	28	47	5½	8.6

Assay performed as described in text. Incubation time of 40 minutes and contact time of 30 minutes with a cystine concentration of M/2000.

hours storage of a 10% homogenate of rat liver at 8°C resulted in complete loss of activity, but on freezing a similar preparation and maintaining it at −5°C for 12 hours, no losses were observed. It is quite evident, therefore, that the lower the temperature, the more stable is the enzyme and even a few degrees above zero is sufficient for total inactivation.

The dilution of the homogenate to be dialyzed was another factor which had to be taken into account. Curves II and III of Fig. 1 illustrate the changes in total activity resulting from the dialysis of a 2% and a 10% homogenate respectively at 0°C. The 10% homogenate showed no loss in activity while the 2% homogenate was about 50% inactivated in 7 hours, indicating that if the total concentration of protein was reduced, the inactivation increased. These results are similar to those obtained by Nelson and coworkers[8,9] for purified enzymes. These workers showed that the spontaneous inactivation which occurred in dilute solutions of tyrosinase and ascorbic acid oxidase could be reduced by adding solutions of an inert protein, such as gelatin. The above experiments indicate that the dialysis of a 10% homogenate at 0°C would result in little or no loss of succinoxidase activity during the course of 6 hours.

In order to compare the rates at which the C-4 acids were removed under varying conditions and times of dialysis, a method for expressing the observed data was selected in which a rate of change in the inhibition was computed. This value furnished a ready means of comparing results since it was proportional to the rate of disappearance of C-4 acids per unit time. The results included in Table I were calculated to give the average percent increase in the percent inhibition per hour over the time interval indicated, based on the initially obtained percent inhibition. Such a comparison is valid since the rate is approximately a linear function of the time.

We were concerned in these experiments (Table I) primarily with the speed of disappearance of the factors which influenced the degree of inhibition. Exp. 1 and 2 show clearly how the change in inhibition varied with temperature. At the higher temperature dialysis was more rapid as shown by the higher numerical change in the inhibition. Similarly, Exp. 2 and 3 illustrate the changes observed with a 2% and a 10% homogenate respectively and demonstrate that in the more concentrated homogenates, the substances affecting the inhibition were removed more slowly.

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[8] Adams, M. H., and Nelson, J. M., *J. Am. Chem. Soc.*, 1938, **60**, 2472.

[9] Lovett-Janison, P. L., and Nelson, J. M., *J. Am. Chem. Soc.*, 1940, **62**, 1409.

Since all previous experimental work had been performed on homogenates of mouse kidney, rat liver homogenates were subjected to similar treatment for comparative purposes. Contrary to previous preliminary observations,[1] these two tissues seemed to differ widely, both in the degree of inhibition and in the rate of disappearance of the factors affecting the inhibition. The rate for rat liver homogenates was almost double that of a homogenate of mouse kidney, and in addition, the extent of inhibition was just about half, 42% and 19% inhibition respectively.

It was not evident whether the results observed were due solely to dialysis or whether the series of reactions already known to function in the homogenate[1] was a contributing factor. Parallel experiments were performed in which a portion of the homogenate was placed in a tube and maintained under the same conditions as the portion dialyzed. In the case of mouse kidney, a comparison of Exp. 3 and 4 of Table I shows that on storage the removal of the substances interfering with the normal inhibition of the enzyme occurs slowly but by no means as rapidly as on dialysis of the homogenate. On the contrary, in rat liver preparations dialysis did not increase the removal of such substances over storage alone (Exp. 6 and 7 of Table I).

An increase in inhibition on storage had been previously attributed to a destruction of the C-4 acids through the cozymase-linked malic dehydrogenase.[1] To test this hypothesis further and to see if the postulated reactions were functioning in the cold, a sample of mouse kidney homogenate was stored at 0°C and cytochrome *c* and coenzyme I[||] were added (Exp. 5 of Table I). The resultant rate of increase in inhibition with time was doubled over that obtained without addition of these two substances and was numerically similar to that obtained with rat liver preparations. A possible explanation of the differences secured with these two tissues is that mouse kidney has a lower concentration of coenzyme I and cytochrome *c* than rat liver. Thus on homogenization these substances are reduced to ineffectual concentrations in the case of the mouse kidney but still remain active in the rat liver homogenates.

A major factor in limiting dialysis as a rapid method of securing homogenates free of substances which interfere in the determination of the inhibition is that the inhibition varies inversely as the log of the C-4 acid concentration.[1] Therefore a large decrease in the C-4 acid concentration results in a small increase in the inhibition. Many of the data presented indicate a loss of 80% to 90% of the original concentration of C-4 acids in the course of about 6 hours dialysis. Dialysis can thus be used as a method of reducing the concentration of substances such as the C-4 acids which interfere with the inhibition reaction.

Summary. 1. The inhibition of the succinoxidase system by cystine can be increased by dialysis of the tissue homogenate. 2. Temperatures above 0°C and dilution of the tissue homogenates (below 10%) favor the spontaneous inactivation of this system.

[||] We are indebted to Merck and Company, Inc., Rahway, N.J., for a generous gift of cozymase.

14838 P

Production of Small Colony Variants of *Staphylococcus aureus* by the Action of Penicillin.*

Guy P. Youmans, Elizabeth H. Williston, and Marcia Simon. (Introduced by Alex. A. Day.)

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The production of stable small colony variants from one strain of *Staphylococcus albus* by the action of penicillin was reported by Schnitzer, Camagni, and Buck.[1] In view of the fact these workers found their small colony variants to be penicillin resistant it was thought advisable to determine whether penicillin would have the same action on pathogenic strains of *Staphylococcus aureus*.

Methods. Ten recently isolated, hemolytic, coagulase positive, pathogenic strains of *Staphylococcus aureus* were employed. These strains were all purified before exposure to penicillin by 3 successive single colony isolations.

Serial dilutions of a solution of the sodium salt of penicillin sterilized by filtration were made aseptically in tubes containing 5.0 cc of nutrient broth, each dilution being half the preceding one. The concentration of penicillin in the first tube of each series was one unit per cc. Each tube, including suitable control tubes containing no penicillin, was then inoculated with 0.1 cc of a 24-hour broth culture of the organism to be tested. Readings of growth inhibition were made following 24-hour incubation at 37°C and agar plate subcultures were then made from the control tubes and from a sufficient number of tubes in each series of dilutions to overlap, by 2 or 3 tubes, the zone of growth inhibition. These subcultures were repeated at 2- to 3-day intervals over a period of 7 to 10 days. The agar plates were incubated at 37°C and examined daily for a period of not less than 4 days.

Results. Small colony variants were obtained from 9 of the 10 strains in from 2 to 9 days following the initial exposure to penicillin. None were found in the controls. The susceptibility of the 10 strains to penicillin varied considerably and there was no absolute relation between the concentration of penicillin and the production of small colony variants. Instead, these variants always appeared in the concentration of penicillin which markedly inhibited but did not completely prevent growth. They were never found in more than 2 tubes in a series of dilutions. Furthermore, the concentrations of penicillin in which the appearance of small colony variants was most pronounced were usually those in which there was no evidence of growth following 24-hour incubation but which gradually became turbid following further incubation. These findings are similar to those previously reported on the production of small colony variants by inorganic salts.[2]

The number of small colony variants obtained varied from strain to strain and from tube to tube. Sometimes only a few would be found interspersed among normal *Staphylococcus aureus* colonies, at other times nearly all of the growth on a plate would consist of these variants.

All of the small colony variants obtained were of the unstable type and had the typical characteristics previously described for this form.[2] The rapid reversion of these forms made it impossible to test their penicillin sensitivity but the manner of their production would suggest a greater resistance than the normal form of the organism.

These results show that penicillin is an effective agent for the production of small colony variants of *Staphylococcus aureus* and since they appear most readily in concentrations of penicillin which are almost completely inhibitory, the results further indicate the advisability of using maximal rather than minimal doses of penicillin for the clinical treatment of staphylococcal infections.

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* Part of the penicillin employed was furnished by the Penicillin Research Committee, Northwestern University Medical School.

[1] Schnitzer, R. J., Camagni, Lillian J., and Buck, Margaret, Proc. Soc. Exp. Biol. and Med., 1943, **53**, 75.

[2] Youmans, Guy P., and Delves, Edna, *J. Bact.*, 1942, **44**, 127.

Summary. Unstable small colony variants were readily produced from nine out of ten strains of *Staphylococcus aureus* upon exposure to penicillin.

14839

Observations on the Antagonism Between Heparin and Trypsin.*

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From the Departments of Pharmacology and Chemistry, Northwestern University Medical School, Chicago.

It has been stated that following anaphylactic shock there may be more than the normal amount of antitrypsin in the blood.[1,2,3] The incoagulability of the blood in anaphylaxis has been explained by the discovery that it contains substantial amounts of heparin,[4] and reports that heparin has antitryptic properties[5,6] have led to the suggestion that this substance may be responsible for the increase in the antitryptic activity of such blood.

We have observed that heparin inhibits the histamine releasing effect of trypsin on rabbit blood cells,[7] and that it protects rats from the lethal effects of intravenously injected trypsin.[8] Our observations were interpreted as supporting the view that heparin has a specific antitryptic action. However, the recent demonstration that the blood of heavily heparinized dogs has no increased antiproteolytic activity[9] necessitates a reconsideration of such a view, and has led to the additional studies reported here on the antagonism between heparin and trypsin.

The intravenous injection of heparin (10-15 mg per kilo) did not modify the fall in blood pressure produced by the intravenous injection of trypsin (0.1-1.0 mg per kilo) into anesthetized dogs (4 experiments). Nor did the incubation of heparin with trypsin for various periods of time prior to injection alter its depressor activity. On the other hand, the trypsin preparation used ("Difco") was inactivated when incubated with blood serum, which is known to be antitryptic. These results, therefore, seem valid and suggest that heparin is not a specific antitryptic agent.

Accordingly, it was thought necessary to reinvestigate the *in vitro* antitryptic action of heparin by means of a standard enzyme method. The Anson-Mirsky hemoglobin digestion method was used. Mixtures of heparin and trypsin were incubated at 25°C. At various intervals a 5 cc fraction of the mixture was added to the hemoglobin substrate and the tryptic activity determined. Mixtures of trypsin with serum were incubated and

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* This investigation has been made with the assistance of a grant from the Clara A. Abbott Fund of Northwestern University.

We are indebted to Roche-Organon, Inc., for the supply of Heparin (Liquaemin) used in our experiments.

The fastusol was obtained from Central Scientific Co., Chicago, Ill., as Chlorazol Fast Pink B.

[1] Jobling, J. W., Petersen, W., and Eggstein, A. A., *J. Exp. Med.*, 1915, **22**, 401.

[2] Teale, F. H., and Bach, E., *Proc. Roy. Soc. Med.*, 1919-1920, **13**, pt. 3, sect. Path., p. 5.

[3] Burdon, K. L., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 24.

[4] Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

[5] Horwitt, M. K., *Science*, 1940, **92**, 89.

[6] Glazko, A. J., and Ferguson, J. H., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 43.

[7] Dragstedt, C. A., Wells, J. A., and Rocha e Silva, M., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 191.

[8] Dragstedt, C. A., Wells, J. A., and Rocha e Silva, M., *Fed. Proc.*, 1942, **1**, 149.

[9] Grob, D., *J. Gen. Physiol.*, 1943, **26**, 405.

TABLE I.
Influence of Various Agents on Proteolytic Activity of Trypsin.

Inhibiting agent (vs. trypsin 2.25 mg)		pH†	Activity* before inhibitor was added	Activity* remaining after incubation with inhibitor for						
				5 min	10 min	15 min	20 min	25 min	30 min	60 min
Heparin	1.25 mg	7.5	1.07	1.24	1.24	1.15	1.35	1.30	1.20	
”	12.50 ”	7.5	0.92	1.10	1.20	1.10	1.10	1.12	1.20	
”	12.50 ”	6.3	1.07	1.22	1.20	1.20	1.30	1.20	1.20	
”	12.50 ”	7.5	1.75		2.10		1.90		2.05	2.05
Dog Serum	5.0 cc									
+ Heparin	12.5 mg	7.6	1.75		0.08		0.10		0.09	0.10
Dog Serum	5.0 cc	7.6	1.75		0.03		0.00		0.00	0.00
” ”	2.0 ”	7.6	1.35						0.00	0.03
” ”	0.4 ”	7.6	1.35						0.66	0.60
Fastusol	12.5 mg	7.5	0.95	1.15	1.40	1.07	1.08		1.08	

* Tryptic activity expressed as Anson-Mirsky Units × 10^4.
† pH controlled with phosphate buffer.

tested in the same way so that the effects of a known inhibitor could be seen. Mixtures of trypsin with serum plus heparin were also studied to determine whether or not heparin augments the inhibition due to serum alone. The apparent increase in tryptic activity seen following the addition of heparin or fastusol is unexplained, but is thought to be due to an action of these agents on the hemoglobin substrate. It was not due to the addition of color-producing substances since blank determinations have taken this into consideration.

In view of our inability to confirm the observation that heparin can inhibit to any substantial degree the proteolytic action of trypsin *in vitro,* it was decided to determine whether heparin has a prophylactic value against the injection of trypsin into any other animal than the rat, in which its antagonistic action to trypsin was quite marked. It was found that it has a similar prophylactic value in guinea pigs. Injection of varied doses of a trypsin preparation (Difco) into 33 normal animals indicated that the LD_{50} was approximately 66 mg per kilo and that 80 mg per kilo would kill 100% of animals. In 30 animals injected with heparin (45 to 130 mg per kilo) 10 minutes previously, the indicated LD_{50} of the same trypsin preparation was 114 mg per kilo and the 100% lethal dose approximately 200 mg per kilo. The results indicate an effect comparable to that seen in the rat.

If the protective effect of heparin is not due to a specific antitryptic action the possibility that it might be due to its anti-coagulant action suggests itself. Accordingly, the prophylactic value of another anti-coagulant (Fastusol) was examined. It was found that Fastusol in doses of 24 to 90 mg per kilo raised the LD_{50} of a trypsin preparation in rats from 140 mg per kilo to 250 mg per kilo. As Fastusol did not show marked anti-tryptic action when tested by the Anson-Mirsky procedure (see Table I), these results support the view that the anticoagulant action is important. Additional support for this is provided in that postmortem examination of both guinea pigs and rats dying from trypsin injections showed some hemorrhagic infarction of the lungs and a dilated right heart. These findings indicate that intravascular clotting with pulmonary embolism may be a factor in the lethal action of trypsin in these animals, even though no gross evidence of intravascular clotting was found. As it is known that the clotting action of trypsin on blood can be prevented by heparin,[10] the prophylactic value of heparin in these cases can be explained without requiring that heparin have a specific antitryptic action.

As yet no explanation for the effect of heparin in inhibiting the release of histamine from rabbit blood cells by trypsin has been offered.

Summary. Heparin does not appear to be a specific antitryptic substance since it prevents neither the fall in blood pressure produced by trypsin in the dog nor the proteolytic activity of trypsin on a hemoglobin substrate. However, heparin does protect guinea pigs against the lethal effects of intravenously injected trypsin.

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[10] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 33.

The observation that fastusol, like heparin, protects rats against the lethal effects of trypsin is compatible with the notion that such a protective action on the part of heparin is related to its anticoagulant properties.

14840 P

"Lipid-Steroid" Fractions of Mouse Adrenal Lipids.

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The amount of the lipid material of the mouse adrenal gland was demonstrated by Vicari[1] to vary with the strain used. The technic and method applied was histological in nature. This report deals with initial experiments in separating the lipid material of adrenal glands of different strains of mice into 2 fractions. The purpose is to see wherein lies the true difference between strains, that is, whether the variations hold true for both fractions. The 2 fractions are designated "lipid," materials soluble in petroleum ether. These include cholesterol and cholesterol esters. The other fraction is "steroid" material not soluble in petroleum ether.

Material. Three inbred strains of mice were used. The histories of these are as follows: *C57black,* inbred for over 50 generations with a 1% incidence of mammary gland tumor in breeding females, and a 0% incidence in virgin females; *dba,* inbred for over 50 generations with a 70% incidence of mammary gland tumor in breeding females and a 50% incidence in virgin females; *ce* strain, inbred for 35 generations with less than 1% incidence of mammary gland tumor in breeding females and none in virgins. The animals used for these experiments were normal virgin females 49 days old. Tumors first appear in the tumor strains at 6 months old for breeding females and older for virgin females. The average individual body weight was 19.5 g. None of the animals used had tumors.

Procedure.† For each test 4 adrenals from 2 littermates were used. Two tests were made for each group. The glands were trimmed, weighed, homogenized with a 1:3 mixture of absolute alcohol and acetone A.R. The homogenate was refluxed for 12 hours on a steam bath. The petroleum extract yielded the "lipid" fraction while the 70% alcohol extract yielded the "steroid" fraction. Each extract was evaporated to dryness on a steam bath and the residue taken up in a 1:3 alcohol-acetone mixture and appropriate aliquots taken for analysis by the Van Slyke manometric method[2] for determination of carbon.

Results. Per adrenal gland the *C57black* strain has the highest amount of total carbon, "lipid" and "steroid" fraction, 0.73 mg, 0.61 mg, 0.12 mg respectively, while the *dba* strain, the lowest amount, 0.37 mg, 0.36 mg, 0.01 mg respectively. The *ce* strain has values in

TABLE I.
"Lipid" and "Steroid" Fractions in Adrenal Glands of Mice.

	Amount of Carbon per mg of Adrenal Gland		
Strain	*C57 black*	*ce*	*dba*
	mg	mg	mg
Avg gld. wt.	2.75	4.02	2.80
	mg	mg	mg
Total carbon	0.26	0.15	0.133
"Lipid" Fr.	0.22	0.12	0.129
"Steroid" Fr.	0.04	0.03	0.003

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* Finney-Howell Foundation Medical Research Fellow.

[1] Vicari, E. M., *Anat. Rec.*, 1943, **86**, 523.

† This procedure was devised by Dr. K. Dobriner of the Memorial Hospital, New York City. We are deeply indebted for his assistance and suggestions.

[2] Van Slyke, D. D., Page, I. H., and Kirk, E., *J. Biol. Chem.*, 1933, **102**, 635; Van Slyke, D. D., and Folch, I., *J. Biol. Chem.*, 1940, **136**, 509.

between, 0.60 mg, 0.48 mg and 0.12 mg respectively. Since the *ce* adrenal gland was heavier than the *C57black* and *dba's* weight, which were the same, the amount of carbon was calculated per milligram of adrenal tissue. These calculations are given in Table I. The ratio of the "steroid" to the "lipid" fraction is 1:38 for the *dba,* 1:5 for the *C57black,* and 1:4 for the *ce* strain.

The 3 strains vary not only in their amount of total lipids but also in their amount of the two fractions. The chemical analysis of the adrenal glands of genetic strains of mice indicate the existence of different amounts of "lipid" and "steroid" contents that appear at present to be associated with the incidence of mammary gland tumor.

This work is being continued and extended and the present conclusions are naturally of a preliminary and tentative nature subject to further study.

14841

Effect of Increased Protein Intake on Nitrogen Retention in Hypoproteinemic State (Meleney Ulcer).*

RICHARD L. VARCO. (Introduced by O. H. Wangensteen.)
From the University of Minnesota, Minneapolis, Minn.

Information pertinent to the protein intake requirements and some of the results obtained by means of daily feedings of large quantities of protein from two sources to a patient with a Meleney type ulcer of the anterior abdominal wall, comprise the subject matter in this presentation. K.P., age 33, University Hospital Case 740032, had a Meleney ulcer with the edges subcutaneously dissected which traversed the abdomen from one iliac crest to the opposite side, had engulfed the umbilicus, and extended nearly to the costal margin. The progress of the lesion had been associated with a large weight loss, anorexia, febrile episodes, and general bodily and mental deterioration. She was reluctant even to eat when fed by ward attendants, nor would she cry out for medication when in distress. A continuous 24-hour-a-day intragastric drip feeding program with 100 g of powdered bovine plasma protein in 500 cc of water and sufficient quantities of diet to approximate 4000 calories daily were given. After one week, these quantities were increased to 200 g of powdered bovine plasma protein and enough diet to provide more than 4800 calories daily. The hemoglobin value percentage of plasma proteins and albumin fraction were secured at the start of the feeding, during the course of the project, and at its conclusion. Blood volumes were obtained at the start and finish. This data has been recorded in Chart 1. The most striking rise in the plasma protein value occurred in the albumin fraction which increased from 2.7 g% on 6-19-44 to 3.7 g% on 7-12-44. While the hematocrit rose from 32.5% on 6-19-44 to 35.5% on 7-19-44, the blood volume was enlarged from 3.93 liters to 5.5 liters during the same interval. It is obvious that plasma protein regeneration has been effective by this feeding schedule. The magnitude of the quantities regenerated is hardly appreciated until the factor of increased blood volume is brought into the calculations. It is then apparent that the total albumin content of the plasma volume has been nearly doubled during the test period. Such data serves to emphasize both the hazard of submitting such a patient with a reduced blood volume to any additional blood-letting and the wisdom of providing for restoration of vascularly retained fluids before surgery is undertaken. Interesting also, is the seeming failure of the body to act effectively on avail-

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* The researches presented here were supported by grants of the Augustus L. Searle Fund for Experimental Surgical Research, the Citizen's Aid Society, and the Graduate School of the University of Minnesota.

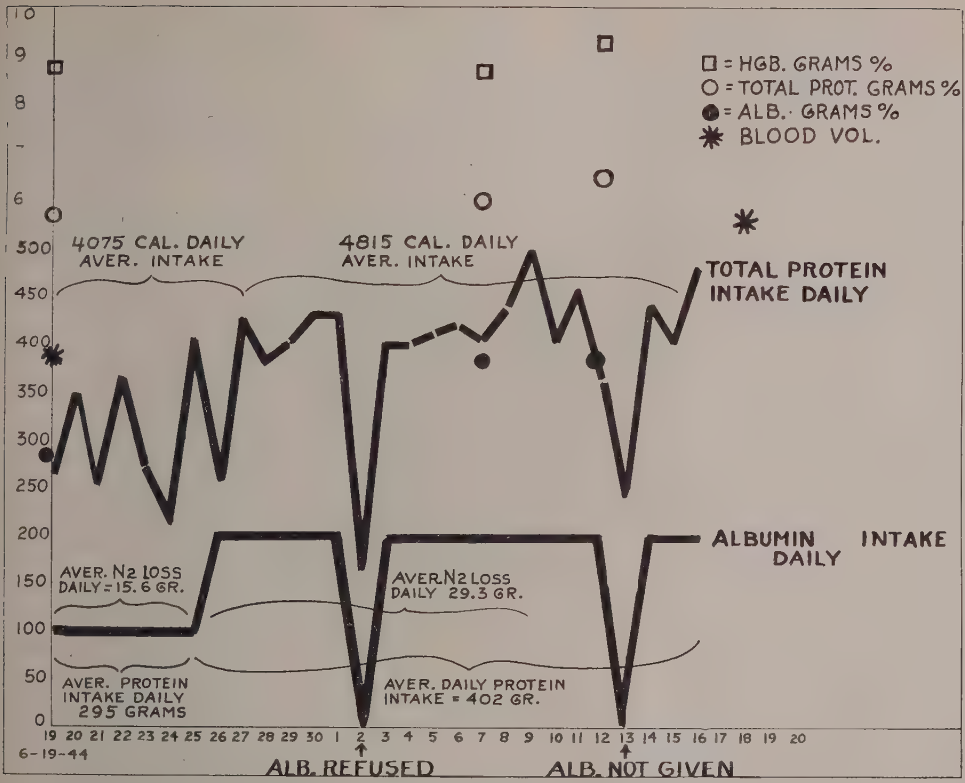

Chart 1.
Effect on Plasma Protein Value of Adding Powdered Bovine Plasma Protein to Diet of Patient with Meleney Ulcer.

Calculation of Albumin Gain.

Blood volume 6-19-44	3.93	liters
Hematocrit 6-19-44	32.5	%
Plasma albumin fraction 6-19-44	2.7	g %
Calculated circulating plasma albumin	71.62	g
Blood volume 7-19-44	5.5	liters
Hematocrit 7-19-44	35.5	%
Plasma albumin fraction 7-12-44	3.7	g %
Calculated circulating plasma albumin	131.26	g
Calculated gain in circulating albumin	59.64	g

able protein, *i.e.*, there is a progressive rise in the intake-conversion ratio. That is, increasing the protein content of the diet from the average of 295 g to 402 g daily actually contributed but 20 g additional daily to the protein retained by the body since the remaining 85 g were lost through an increased daily excretion of urinary nitrogen. In the presence of a real need for protein fabrication the mechanisms controlling this phase of metabolism find limitation rather in their own processes of conversion, rather than in the digestive and absorptive abilities of the intestine. This impression is corroborated by very low values obtained in analysis of the stool content for protein nitrogen.

Discussion. The results of Madden[1] and Whipple suggest that powdered bovine plasma proteins would be an excellent source of material for promoting plasma protein regeneration. A liberal supply has been made available for investigational purposes.†

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[1] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

The results of this study, an earlier one, and unpublished data, suggest strongly that it is therapeutically feasible to raise the plasma protein values under the conditions outlined. The real elevation of the plasma albumin fraction and the total quantity of plasma albumin, due to an increase of the blood volume, appear significant. It is suggested by these data and similar observations on other patients being prepared for operation by feeding of a diet rich in protein and carbohydrate and low in fat, that the intolerance to surgical trauma exhibited by such chronically wasted patients, is related to just such a contracted blood volume.

Conclusion. Feeding powdered bovine plasma protein to a patient made hypoproteinemic by a Meleney ulcer, was associated with an elevation of the plasma protein value. A large measure of this increase occurred in the albumin fraction. A daily intake of more than 500 g of protein was well tolerated by an ill patient.

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† Provided by Armour and Company, Chicago.

14842

Effect of a Chromatin Derivative on the Healing of Skin Wounds.

A. MARSHAK AND A. C. WALKER.
*From the Radiation Laboratory, University of California.**

Experiments which are to be reported elsewhere showed that chromatin given intravenously produced a marked stimulation in the rate of mitosis in the regenerating liver of the rat.[1] We wish here to describe preliminary results obtained when a fraction derived from chromatin was applied to skin wounds.

Rat liver was perfused with 0.9% of NaCl and blended into 4 volumes of 0.9% of NaCl for 2 minutes in a Waring blendor. Three-tenths molar NaOH was added during the blending in amounts sufficient to bring the pH to 7.4. The suspension was treated by the method of Claude and Potter[2] in order to isolate the filamentous substance they have called chromatin. The chromatin was lyophilized and extracted with boiling 50% acetone-50% alcohol. The fat-free chromatin was then extracted with 0.9% of NaCl. The extract was then precipitated with acetone in which it was kept overnight. The clear supernatant solution had a bright yellow color. After decanting most of the acetone, the white precipitate was transferred aseptically to sterile test tubes in which it was lyophilized and sealed *in vacuo*. The 2.4 mg of dried material in each tube was taken up in 2 ml of saline for use on the rat wounds.

The wounds were made as described in the preceding paper. Controls were treated exactly as were the test animals, except that sterile saline was applied instead of the chromatin extract. With a hypodermic syringe, 2 or 3 drops were placed on the wound so that they formed one large drop filling the skin defect.† With a brush, a solution of methacrylate polymer in toluene was then applied over the drop and the adjacent normal skin. In the first 2 series of animals some of the wounds became infected. It was discovered that in these cases there had been leakage of the saline or test solution onto the normal skin so that the wound was not actually completely

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* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California.

[1] Marshak, A., and Walker, A. C., unpublished.

[2] Claude, A., and Potter, J. S., *J. Exp. Med.*, 1943, **77**, 345.

† When the chromatin derivative was applied to wounds in which there was bleeding, the bleeding was stopped almost instantaneously.

sealed, although it seemed to be so on casual inspection. This difficulty was eliminated by painting the skin around the wound with the methacrylate polymer before the saline or test solution was applied. Following this procedure none of the wounds became infected. There was no evidence of irritation or inflammation following the application of the chromatin fraction.

For the first two series the following results were observed on the fifth postoperative day:

Granulation Tissue on the Fifth Post-Operative Day.

	Control		Experimental	
Granulation				
Absent	12		3*	
Present	3	<1/10 wound area	13	¼ to ⅔ wound area

*Wounds badly infected.

Of 15 controls only 3 showed any granulation on the fifth day. It appeared as a band 1 mm or less in width, about the periphery of the wound. On the other hand, only 3 of the 16 chromatin-treated wounds failed to show granulation tissue covering at least ¼ of the wound, and these 3 were badly infected.

In the third series in which none of the wounds became infected, no granulation appeared in the 4 control wounds until the fifth day. Of the 5 experimental animals, small islands af granulation appeared in 2 of them as early as the second day, in another on the third day, and by the fourth day all had granulation tissue over ¼ to ⅔ of the wound area. The granulation tissue in the experimental animals was bright red as compared with the pale red or pink of the controls and in microscopic section was clearly more vascular. Although the granulation tissue appears earlier in the treated wounds, the amount of such tissue formed is no greater than in the controls. After epithelialization was complete, the elevation of the treated wounds above the surrounding skin was no greater than in the controls.

Circumstances made it impossible to continue these experiments. However, the results obtained seem sufficiently promising to warrant further study.

We are indebted to Dr. E. L. Howes for testing the effect of whole chromatin on rabbit wounds and tissue cultures. In a personal communication he has reported that following intravenous administration of the chromatin, there was an optimum rate of healing of wounds of the rabbit's ear. This substance also increased the rate of growth of fibroblasts in tissue culture.

Summary. Following the application of a chromatin fraction to skin wounds of the rat, granulation tissue appeared one to three days earlier than in the controls. On the fifth postoperative day, several times as much of the surface of treated wounds was covered with granulation tissue as in the controls. There was no evidence of inflammation or irritation, and an excess of granulation tissue was not found.

14843

Healing of Experimental Skin Wounds.

A. Marshak.

*From the Radiation Laboratory, University of California.**

In the methods for the study of wound healing which have previously been described, there are inadequacies and sources of error which limit the usefulness of the procedures used. Carrel[1,2] used the rate of closure of square skin defects as an index of healing,

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* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California.

[1] Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

[2] Carrel, A., and Hartman, A., *J. Exp. Med.*, 1916, **24**, 429.

and by mathematical analysis, du Noüy[3,4] extended the method to apply to wounds of irregular shape. However there was no experimental segregation of two processes involved in wound closure, *i.e.*, contraction and proliferation. As a consequence, on the basis of du Noüy's equation for normal healing, a small wound would be expected to heal instantaneously which of course is not in accord with observation. Howes, Sooy, and Harvey[5,6,7,8] determined rate of healing by measuring the tensile strength of linear wounds. Although they mention the desirability of an experimental separation of the "contraction and epidermization processes," their method yields little or no information on changes occurring in the first 5 days of healing. More recently, Howes[9] used the rate of extension of a small island of epithelium in a large, square skin defect as an index of healing rate. This method assumed that contraction would not affect the rate of epithelial extension but did not provide a means for controlling or analyzing such contraction. In studying the effect of various substances on wounds, it was necessary in all of these methods to incorporate the agent in an ointment which introduced further complications. Since gauze bandage dressings were used, it was difficult or impossible to keep the wound aseptic, especially when repeated inspection and measurement were necessary. In an effort to avoid these difficulties the following technic was devised.

A stainless steel tube was ground at one end to make a circular cutting edge 6.6 mm in diameter.[†] With it a depression was made in the skin of the back of the neck of the anesthetized rat, and along this line the skin was cut with a pair of iridectomy scissors through the dense connective tissue of the dermis, but not through the loose connective tissue of the hypodermis. The skin thus circumscribed was then lifted off without any dissection. With care and practice the thickness of the connective tissue left in place was surprisingly uniform. There was usually no bleeding and when it did occur it was easily controlled with small swabs so that the wound contained no blood clots. A thick solution of methacrylate polymer[‡] dissolved in toluene was then dropped onto the wound from a small brush and the adjacent skin painted with the same solution to make a coat completely covering the wound and extending 3 to 5 mm beyond it. In 15 minutes the coat had dried sufficiently so that the animal could be returned to its cage. No contraction occurred on drying as was the case with collodion or some other plastics. To keep the animal from pulling the coat off, its hind paws were covered with adhesive tape.

The coat formed did not adhere to the wound tissue but did cling to the surrounding normal skin so that a completely protective covering was provided which at the same time eliminated tissue damage due to adhesion and friction. If, before the plastic was applied the surface of the wound was allowed to dry, adhesion did occur, and if there was moisture on the adjacent normal skin, the plastic did not adhere. There was no vascular reaction

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[3] du Noüy, P. L., *J. Exp. Med.*, 1916, **24**, 451.

[4] du Noüy, P. L., *J. Exp. Med.*, 1919, **29**, 329.

[5] Howes, E. L., Sooy, J. W., and Harvey, S. C., *J. A. M. A.*, 1929, **92**, 42.

[6] Howes, E. L., and Harvey, S. C., *New England J. Med.*, 1929, **200**, 1285.

[7] Howes, E. L., and Harvey, S. C., *Yale J. Biol. and Med.*, 1930, **2**, 285.

[8] Harvey, S. C., and Howes, E. L., *Ann. Surg.*, 1930, **91**, 641.

[9] Howes, E. L., *Surg., Gyn., and Obst.*, 1943, **76**, 738.

[†] A more complicated instrument was constructed in which the cutting tool protected by a guard of lucite could be rotated by means of a flexible shaft connected to a motor. The depth of the cut could be regulated by adjusting a vernier scale on the hand piece of the tool. It was not used in these experiments since it offered no advantages over the simple tube in the excision of the cervical skin of the rat. It is mentioned, however, since it did prove to be more useful in the excision of portions of skin from the abdomen or back which is much more firmly attached to the hypodermis.

[‡] When isobutyl methacrylate polymer was used, it was found necessary to apply a second coat on the day following the operation to prevent its cracking. This was not necessary when a mixture of equal parts of the isobutyl polymer and interpolymer of normal and isobutyl methacrylate was used. We are indebted to the E. I. du Pont de Nemours Company for supplying us with these materials.

and no visible exudate. By the fourth or fifth day following the operation some of the plastic coats began to come loose along the edges. These edges were then repainted with the same solution of the plastic and allowed to dry before the animal was returned to the cage. In this way, with relatively little attention, the wounds were kept sealed and protected until they were completely epithelialized. The changes which took place could be observed and measurements made without disturbing them in any way.

The plastic acted as a splint which prevented contraction of the wound. The marked contraction which occurs in unsplinted wounds[1,2,6] was not observed under these conditions. That contraction can occur after the wound is completely filled with granulation tissue may be demonstrated by removing the plastic coat at that time (8th day post-op.). On the following day, the diameter of the wound is reduced and a papilla of granulation tissue 2 to 4 mm high appears in the center of it.

A further advantage of the method is that the effect of aqueous solutions may be determined without having to incorporate the solution into an ointment which would complicate interpretation of the results observed. An example is given in the preceding paper on the effects of a derivative of chromatin.

The use of the plastic dressing made it possible to study the uptake of radioactive phosphorus (P^{32}) and radioactive sulfur (S^{35}) by the wound during the healing process. The data obtained in these experiments will not be presented here but are mentioned as an additional example of the usefulness of the plastic dressing.

Although the plastic solution produces no reaction in skin wounds of the rat and rabbit, it was found unsuited to the study of human skin wounds since it stimulated the formation of copious amounts of serosanguinous exudate. It may have some practical use, however, since it has been found that when applied to cuts, bruises, and small burns of the hand on the second or third day there is no exudation. Since the plastic does not contract, as does collodion for example, an entire finger may be coated. It has been found to adhere and protect the wound for 3 to 4 days under conditions (K.P. duty) where a bandage dressing was of little value. By the proper combination of normal and isobutyl polymers almost any degree of flexibility may be obtained.

Summary. The use of methacrylate polymers as a wound dressing in rodents is described and its advantage in the experimental study of wound healing indicated.

14844

Origin of Cardiac Disorders in Thiamine-Deficient Cats.

J. E. P. Toman, G. M. Everett, R. H. Oster, and D. C. Smith. (Introduced by L. S. Goodman.)

From the Department of Physiology, University of Maryland School of Medicine, Baltimore.

Some of the cardiac disorders reported in thiamine deficiency may be neurogenic rather than myogenic in origin.[1,2] The following observations suggest a dual origin of the electrocardiographic signs in the thiamine-deficient cat.

Method. The method used for producing specific thiamine deficiency in cats has been reported previously in connection with a study of the neurological findings.[3] During the development of the deficiency and during the recovery period, limb lead electrocardiograms were taken in 37 unanesthetized animals. The electrocardiograms were recorded with an ink-

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[1] McEachern, D., and Brophy, D., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 75.

[2] Weiss, S., and Wilkins, R. W., *Ann. Int. Med.*, 1937, **11**, 104.

[3] Everett, G. M., *Am. J. Physiol.*, 1944, **141**, 439.

TABLE I.
Incidence of Abnormal Electrocardiographic Findings in Thiamine-deficient Cats.

Abnormality	No. abnormal	Total No. of cats	% abnormal
(1) Prompt response to thiamine			
Bradycardia*	22	37	59
Tachycardia*	11	37	30
Sinus irregularity	32	37	87
Excitement syndrome	17	18‖	95
Ectopic rhythms	5	37	14
(2) Slow response to thiamine			
QRS prolongation†	8	16‡	50
Other QRS changes†	5	16‡	31
T wave changes†	32	37	87
Inversion of T_2	16	37	43
Prolonged systole-cycle constant*	20	27§	74

* Beyond normal limits for entire group, prior control.

† Compared with prior control on same animal.

‡ Excluding those without satisfactory prior controls.

§ Excluding those with low-voltage T-waves.

‖ Excluding those observed only at rest.

writing oscillograph fed by amplifiers having a suitably long time-constant.

Results. Table I summarizes the abnormal electrocardiographic findings. The disorders may be divided into two groups on the basis of their response to thiamine therapy:

1. *Disorders promptly reversible by thiamine injection.* During thiamine deficiency, sinus bradycardia began as early as the second week. Tachycardia was less frequent and began late. Sinus irregularity was common; it was sometimes related to respiration but more often not. Cycle durations varied widely and randomly from beat to beat and bore no simple numerical relation to each other. The irregularity was greatest at lowest heart rates and disappeared when rates were high, as when the animal was excited by handling, or after atropinization, or during the brief spontaneous tonic convulsive seizures characteristic of cats in the critical stage of thiamine deficiency. Irregularity sometimes occurred with heart rates within the normal range, but not in animals anesthetized with pentobarbital (40 mg/kg) even when heart rate was low. In all but one animal examined during the critical stage of thiamine deficiency, handling of the animal or spontaneous seizures resulted in an "excitement syndrome": a brief period of tachycardia was followed by severe bradycardia and irregularity, sometimes with escaped beats of ventricular or low nodal origin Recovery often required many minutes.

Following the intramuscular injection of thiamine (0.5 to 2.0 mg/kg) in the critical stage of deficiency, a relative tachycardia appeared in all but one animal. It coincided with the first improvement in neurological signs (20 minutes at the earliest) and persisted for as long as 24 hours, after which the rate subsided to normal or below. Recovery both from bradycardia and from disorders of impulse formation and rhythm was complete in most animals within 3 days, at which time neurological recovery was also complete. In 3 animals with persistent neurological signs (ataxia and ophthalmoplegia), recovery from the above cardiac disorders was delayed.

Sections of the brain stem of 2 animals sacrificed in the critical stage of deficiency showed vascular lesions restricted to the midbrain, with associated chromatolysis (*cf.* Weiss and Wilkins[2]).

A neurogenic (central nervous) origin of the above described disorders seems plausible. Their prompt reversibility by thiamine therapy demonstrates the specificity of thiamine deficiency in their production.

2. *Disorders not promptly reversible by thiamine injection.* T waves became flattened or inverted in at least one lead in most animals, the change being evident as early as the 10th day (a small percentage of normal cats showed inversion of T_1 or T_3, but T_2 was always normally upright). Inversion was usually terminal, was associated with a flat and prolonged ST segment, and was independent of wide variations in heart rate. It persisted under pentobarbital anesthesia or full atropinization. Such stability was seen also in the abnormally prolonged systole-cycle constant ($K = QT/\sqrt{cycle}$) and in the altered QRS complexes (prolongation, slurring, and notching).

Alterations in this category were slow to disappear (3 days to 2 months) following thiamine treatment, despite prompt neurological recovery. Their persistence suggests myocardial damage, and the combination of signs points to greater involvement of the left ventricle. However, neither gross nor microscopic pathology was evident in hearts of sacrificed animals. Early appearance and late disappearance of signs did not parallel changes in body weight.

Summary. Electrocardiographic changes in cats with acute thiamine deficiency fall into two categories: (1) disorders of rate, regularity, and impulse formation, which yield promptly to thiamine treatment; (2) changes in the ventricular complex, which respond more slowly to treatment.

14845

An Electrophoretic Study of Tetanus Toxoid Prepared on Mueller's Peptone-Free Medium.

PETER MASUCCI AND RALPH J. DE FALCO. (Introduced by L. Earle Arnow.)

From the Medical-Research Division, Sharp & Dohme, Glenolden, Pa.

Recent advances in the study of the complexity of tetanus toxin have been summarized by Smith[1] and Petrie.[2] Smith compared the toxicity of five different tetanus toxins in mice, guinea pigs, and rabbits. She showed that the ratio of the LD_{50} for the rabbit and mouse and the ratio for the rabbit and guinea pig (weight for weight) may show extraordinary and significant variations, whereas the ratio of the lethal dose for the mouse and guinea pig is more or less constant. Her findings indicate that tetanus toxin contains two or more toxic factors and that the mouse and the guinea pig are relatively more sensitive to one and that the rabbit is relatively more sensitive to the other factor. Petrie studied the variable interactions of tetanus toxins and tetanus antitoxins with the object of throwing light upon the cause of the discrepancies that are met when samples of tetanus antitoxin are assayed in different laboratories. His results indicate that crude tetanus toxins are not homogeneous solutions of toxin and toxoid molecules. Whether they are used as antigens for the hyperimmunization of horses or as test-toxins, crude tetanus toxins are to be regarded as mixtures in varying proportions of the "primary" toxin or toxoid molecule and of an antigenic variant of this which is formed during the later stages of the culture growth; preparations of tetanus serum likewise consist of mixtures of varying proportions of the corresponding antitoxins. Differences in mutual neutralizing affinities of these components in a reacting system account for the discrepancies in the titer of tetanus sera when different test-toxins are used for the assays.

The crude tetanus toxins referred to in these papers were prepared from culture media containing Witte, Riedel, or Difco peptone and beef or horse-meat infusion. The tetanus toxoid studied in the present paper was derived from tetanus toxin, prepared on a medium containing only low molecular nutrient materials. The toxoid could be purified and concentrated without chemical treatment.

Materials and Methods. The tetanus toxin was produced on a peptone-free medium according to the method of Mueller and coworkers.[3] It contained approximately 20,000 guinea pig MLD per cc and had an L^+ of 0.04 cc. The conversion of the hydrolysate tetanus toxin to toxoid was also carried out according to Mueller's procedure.[4] The antigenicity was appraised by injecting 0.4 cc of the toxoid subcutaneously into each of 20 mice. At the end of 10 days, the mice were challenged with 20 MLD of tetanus toxin; 70% of the animals survived.

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[1] Smith, M. L., *Bull. of the Health Organization*, 1942-3, **10**, 104.

[2] Petrie, G. F., *Bull. of the Health Organization*, 1942-3, **10**, 113.

[3] Mueller, J. H., Schoenbach, E. B., Jezukawicz, J. J., and Miller, P. A., *J. Clin. Invest.*, 1943, **22**, 315.

[4] Schoenbach, E. B., Jezukawicz, J. J., and Muller, J. H., *J. Clin. Invest.*, 1943, **22**, 319.

The toxoid was concentrated in the cold room by ultrafiltration through an 8% nitrocellulose membrane formed on a 450 cc Coors porous filter balloon flask. The concentration was 100-fold, since 40 liters of toxoid were reduced to 400 cc. The concentrated toxoid was washed twice with two 400 cc portions of distilled water. After the second washing, the filtrate was almost colorless and still gave a positive test for chlorides. The yellow concentrated solution (400 cc) was divided into two 200 cc portions; one was used for various tests and the other was divided into four 50 cc portions and dried by the lyophile technic.

The undried portion was tested for Lf titer, antigenicity, total nitrogen, and phosphotungstic acid precipitable nitrogen. The concentrated toxoid had an Lf titer of 165 per cc compared to an Lf titer of 1.5 to 2 of the unconcentrated toxoid, indicating that essentially all of the LF units had been concentrated by ultrafiltration. The antigenicity was appraised with the fluid concentrated toxoid and with a portion of the same toxoid converted into alum-precipitated material. The fluid toxoid was diluted with 6 volumes of physiological saline solution and 0.4 cc amounts of the diluted material were injected subcutaneously into each of 20 mice. At the end of 10 days, the mice were challenged with 20 MLD of tetanus toxin; all the animals survived. The alum-precipitated material was injected into 10 guinea pigs, in two 0.05 cc doses spaced three weeks apart. The animals were bled four weeks after the last injection; the blood serum was pooled and tested for antitoxin content. The serum contained 4.0 units of antitoxin per cc. The results of the antigenicity tests on the fluid and alum-precipitated material indicated that the antigenicity of the material not only had not been impaired by the method used for concentrating it but had been remarkably and significantly increased.

The concentrated toxoid contained 0.50 mg of nitrogen per cc of which 74% was precipitable by phosphotungstic acid. This might possibly indicate that the concentrated toxoid had not been completely washed free of low-molecular nitrogen-containing substances. To four test tubes were added 1.0 cc of sodium acetate-acetic acid buffer, ionic strength 0.1, at pH 3.6, 4.0, 4.5, and 4.9 respectively. To each tube was then added 1.5 cc of the concentrated toxoid and 0.5 cc of distilled water. Each tube became opalescent. The tubes were placed in the cold room at 5°C overnight. In the morning a precipitate had settled out in each tube. The tubes were centrifuged; the clear supernatant was saved and assayed for total nitrogen. The total nitrogen in the precipitate was calculated. The results were as follows:

pH	% of original N in precipitate
3.6	12
4.0	24
4.5	24
4.9	12

The precipitate in the pH 4.0 tube was dissolved in Na_2HPO_4-NaH_2PO_4 buffer, ionic strength 0.1, pH 7.0, and tested for Lf units. Only 60% of the original Lf units in the tube were recovered in the precipitate formed at pH 4.0. This might indicate that not all of the substance responsible for flocculation is precipitable at pH 4.0 or that two different substances with different isoelectric points are involved.

Analysis of the Tetanus Toxoid Concentrate by Electrophoresis. The material in one of the bottles containing the equivalent of 50 cc of the concentrate in dry form was reconstituted to the liquid state by the addition of 11 cc of distilled water. The resulting solution, which represented 5 liters of the original unconcentrated toxoid, had an intense yellow color.

Dr. Robert C. Warner, United States Department of Agriculture, Eastern Regional Laboratory, Philadelphia, Pa., kindly consented to do the electrophoretic experiment. Doctor Warner reported that the electrophoretic pattern shows three components. The fastest (marked A on the tracing) is present in very small amount and the other two (B and C) are present in roughly equal quantities. The lower patterns on the tracing were obtained after electrophoresis for 3600 seconds at a field strength of 4.50 volts per cm. The upper

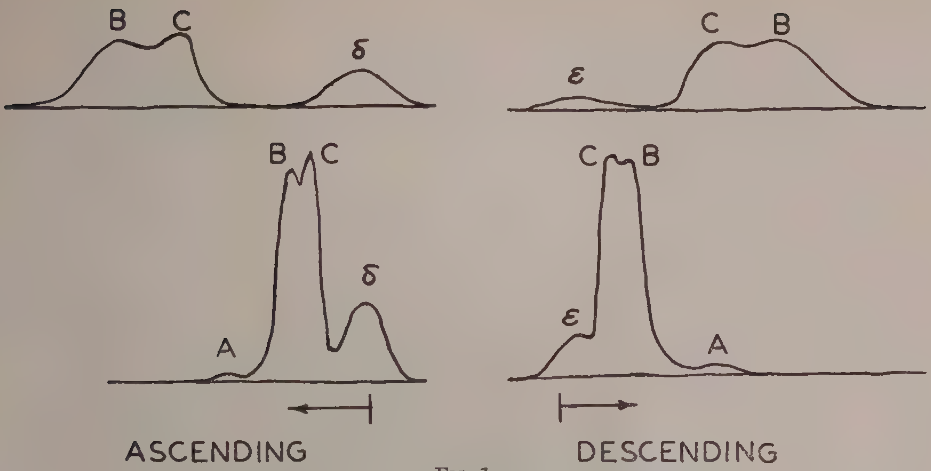

Fig. 1.

The position of the initial boundaries is shown on the tracings by the back end of the arrows at the bottom of each tracing.

patterns were obtained after an additional period of 11,700 seconds at 3.04 volts per cm. The mobility found for component B was 6.5 × 10^{-5} cm^2 per volt per second and for component C was 4.7 × 10^{-5} cm^2 per volt per second in a veronal buffer at a pH of 7.79 and ionic strength of 0.1. The only abnormality noticed was a tendency for the boundaries on the ascending side to be somewhat unstable in that the schlieren bands were curved or irregular at their edges. The position of the initial boundary, the direction of migration and the δ and ϵ boundaries are indicated on the tracing.

Doctor Warner found that the yellow color of the solution interfered with the photography to such an extent that it was not possible to obtain suitable prints from the negatives. Doctor Warner made tracings from the negatives which are reproduced in Fig. 1. Incidentally, the yellow pigment present in the solution migrated with component B in both the ascending and descending channels.

Disregarding component A which is present in very small amount, it would appear that in tetanus toxoid prepared on peptone-free medium and concentrated by the method described, are two major components present in about equal amounts. The yellow pigment present in the solution migrates with only one of the two components.

The question naturally arose, are the two components B and C two different molecular forms or factors of the toxoid as postulated by Smith and by Petrie or is one of these the toxoid (protein) and the other a bacterial protein derived from the autolysis of the bacterial cells during the culture growth. It was hoped that when maximum separation of the boundaries had been effected, the contents of the cell compartments could be removed for the determination of antigenicity and serological activity. Unfortunately, Doctor Warner reported that peaks B and C were not sufficiently separated to make it practicable to attempt the isolation of samples of these components from the electrophoresis cell.

Since the direct method was not available to us, we attempted to answer the question by an indirect approach. Tetanus antitoxic globulin, purified and concentrated by an enzyme digest method, was treated with a suspension of washed, live tetanus organisms. The mixture was incubated one and one-half hours at 37°C and then centrifuged. The supernatant was Seitz-filtered. The filtered antitoxin was again treated with washed, live tetanus organisms, incubated, centrifuged, and Seitz filtered. The Lf units of this absorbed antitoxin were determined 3 times against a tetanus toxoid of known Lf titer. It was then possible to calculate how much of the antitoxin should be added to a given amount of the tetanus toxoid concentrate to obtain flocculation at the optimum point.

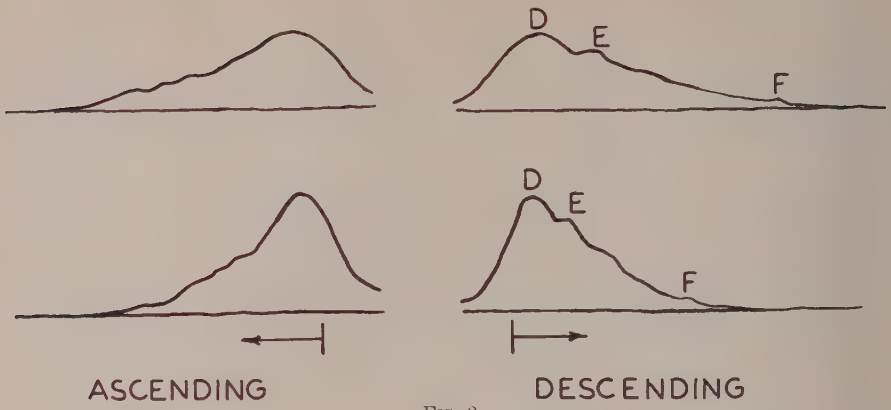

Fig. 2.

The material in another bottle containing the equivalent of 50 cc of the concentrated toxoid in dry form was reconstituted to the liquid state by the addition of 10 cc of distilled water. To 9.5 cc of this (equivalent to 4750 cc of the original toxoid), or approximately 7800 Lf units, were added 7.5 cc of absorbed antitoxin (1020 U/cc). Flocculation occurred within a few minutes. The mixture was incubated at 37°C for 30 minutes and placed in the cold room overnight. The material was then centrifuged and the supernatant was sent to Doctor Warner for a second electrophoretic analysis.

In this experiment the yellow color in the solution was not as intense as in the previous experiment and sufficient contrast was obtained in the photographs to permit prints to be made. Tracings were made from the negative and they are reproduced in Fig. 2. The patterns were obtained after electrophoresis for 8400 seconds (Fig. 2) (lower patterns) and 12,730 seconds (upper patterns) at a field strength of 4.81 volts/cm in a veronal buffer at pH 7.78 and an ionic strength of 0.1.

The patterns show the presence of several components and do not bear any definite relation to the pattern of the original toxoid preparation (Fig. 1). Mobilities have been calculated for three points in the descending pattern, lettered D, E, and F. They were found to be 0.7×10^{-5} cm^2/volt/sec for D; 2.1×10^{-5} for E; and 6.5×10^{-5} for F. Thus, by far the major part of the protein moves with a lower mobility than either peak B or C in the previous experiment. The mobility of the very small peak F, however, corresponds with that of B.

The antitoxin used in the flocculation was a purified and concentrated pseudo-globulin of equine origin and presumably should have been free of bacterial antibodies which are generally attached to the euglobulin fraction. The antitoxin was treated with the live tetanus organisms with the object of removing any anti-tetanic bacterial antibodies if they were present. If this were accomplished, then it might be possible to determine which of the two major components B or C was a bacterial cell derivative if only one of these components reacted with the antitoxin. If both components reacted to form floccules, then neither would be present in the supernatant. The deduction could then be made that neither B nor C was a bacterial cell derivative but both were two different toxoid factors.

In performing the flocculation test, we were well aware that non-reacting proteins present in the antitoxin would be introduced and that these would show up in the supernatant. Antitoxin, in immunologically pure form, has not yet been prepared by methods now in use for fractionating horse plasma. Pappenheimer[5] prepared a diphtheria antitoxic pseudoglobulin which was homogeneous by sedimentation, electrophoresis, and diffusion; yet, only 43.5% of this preparation was specifically precipitable by pure diphtheria toxin.

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[5] Pappenheimer, A. M., *J. Bact.*, 1942, **43**, 273.

TABLE I.
Electrophoretic Analysis of Tetanus Toxoid Concentrate.

Before Flocculation	Supernatant after Flocculation
A—present in very small amount	D—u = 0.7×10^{-5} cm^2/volt/sec.
B—u = 6.5×10^{-5} cm^2/volt/sec.	E—u = 2.1×10^{-5} cm^2/volt/sec.
(yellow pigment)	F—u = 6.5×10^{-5} cm^2/volt/sec.
C—u = 4.7×10^{-5} cm^2/volt/sec.	(present in very small amount).
B and C present in about equal amounts	

There is every reason to believe that the tetanus antitoxin used in this experiment also contained a large proportion of non-reacting protein. If so, then these would appear in the supernatant liquid recovered from the toxoid-antitoxin floccules. That this actually took place is evidenced by the fact that the electrophoretic patterns on the supernatant show the presence of several components and did not bear any definite relation to the pattern of the original toxoid concentrate.

The mobilities of the various components found in the toxoid concentrate before flocculation and in the supernatant after flocculation are tabulated in Table I.

It will be seen from the table that by far the major part of the protein in the supernatant moves with a lower mobility than either peak B or C in the toxoid concentrate before it was allowed to react with the antitoxin. This indicates that the protein with the lower mobilities in the supernatant came from the antitoxin and very likely represents the non-reacting protein present in the antitoxin. The mobility of F corresponds to that of B but the F component is present in very small amount. It appears, therefore, that all of component C and nearly all of component B reacted with the antitoxin to form floccules, an indication, in our opinion, that neither component B nor C is a bacterial cell derivative but that components B and C may be regarded as two different toxoid molecules.

Work is now in progress whereby tetanus toxin, prepared by a procedure which yields a product relatively pure and with a remarkably high MLD, will be subjected to a similar study.

Summary. Concentrated solutions of tetanus toxoid prepared on a peptone-free medium were examined by electrophoresis in a Tiselius apparatus. The concentrate contained three components—A, present in a very small amount, B and C, present in about equal amounts.

The toxoid concentrate was allowed to react with an absorbed tetanus antitoxic pseudoglobulin, the floccules were separated and the supernatant was examined by electrophoresis. The supernatant contained several components, which bore no definite relation to the pattern of the original toxoid concentrate. By far the major part of the protein in the supernatant moved with a lower mobility than either component B or C. The mobility of F which was present in very small amount corresponded to that of B.

The presence of components D and E in the supernatant is ascribed to the non-reacting protein present in the antitoxin. Since all of component C and nearly all of component B was removed by flocculation, this evidence is taken to indicate that there was present in the toxoid concentrate two different toxoid proteins.

Although this work was done with tetanus toxoid and not with the toxin, consideration of the foregoing statements lends support to the views of Smith and Petrie that tetanus toxin (and by analogy tetanus toxoid) is not a homogeneous solution of toxin or toxoid molecules.

We wish to express our gratitude to Dr. Robert C. Warner of the Eastern Regional Laboratory, Philadelphia, Pa., for performing the electrophoretic experiments and for other valuable assistance.

14846

The Occurrence of Anti-Rh_0 Blocking Antibodies in Anti-Rh′ Serums.

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Wiener[1] has reported a new test for isoimmunization to the Rh agglutinogen. So-called "blocking antibodies" were detected in serum specimens from Rh-negative individuals even though agglutinins were absent according to the usual methods of testing. The demonstration of Rh isoimmunization is of sufficient importance to make the blocking antibody test of practical value in diagnostic laboratory work and the performance of this test should not be difficult for those familiar with Rh typing procedures. In the blocking test, agglutination by anti-Rh serums is inhibited by preliminary treatment of the cells with the unknown serum. Normal serums do not interfere with the action of anti-Rh agglutinating serums under the same conditions.

In the present study the blocking antibody test was of value in proving isoimmunization in three cases of erythroblastosis where serum specimens from the Rh-negative mothers showed no Rh agglutinins. The purpose of this report, however, is to call attention to the occurrence of Rh_0 blocking antibodies in Rh subtype testing serums. Rh agglutinins developed in Rh-negative persons are known to differ in specificity in accord with differences in antigenic structure of the Rh agglutinogen and the subtypes of Rh have been classified by Wiener,[2] whose new nomenclature is used in this discussion. Two anti-Rh serums were employed in experiments to demonstrate the presence of blocking antibodies in subtype antiserums. The antiserums, "MF" (Group A) and "Lo" (Group B) were obtained from Rh-negative mothers who had delivered erythroblastotic infants; both produced strong clumping and conformed to the anti-Rh′ subtype in giving about 70% positive reactions with cell suspensions selected at random. Eleven Group O, Rh-positive bloods which gave negative reactions with the two serums were employed. The cells were washed once in saline and used in 2% suspensions which were added in amounts of 1 drop to 1 drop of the antiserums and normal control serum. After preliminary incubation of 30 minutes at 37°C, the tubes were examined for clumping and a drop of anti-Rh_0 testing fluid was added. The tubes were then reincubated and examined for agglutination.

Results of the tests, which are summarized in Table I, showed the presence of anti-Rh_0 antibodies in both of the anti-Rh′ serums which were active for all of the blood suspensions employed. The normal control serum which was used in the same manner did not inhibit clumping.

Wiener[1] has commented on the coexistence of agglutinating and blocking antibodies active in the presence of the same subtypes of agglutinogen. In the present study, blocking

TABLE I.
Blocking Effect of Anti-Rh′ Serums on Anti-Rh_0 Agglutinins.

Serum diluted 1-2 1 drop	2% cell suspensions 1 drop	Anti-Rh_0 serum diluted 1-2 1 drop
Anti-Rh′, "MF" Anti-Rh′, "Lo"	Tubes incubated 30 minutes at 37°C; 11 specimens tested; no clumping noted.	Tubes reincubated; complete or marked inhibition of agglutination with all specimens.
Normal, Rh negative		Strong clumping with all specimens.

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[1] Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 173.

[2] Wiener, A. S., *Science*, 1944, **99**, 532.

antibodies (anti-Rh_o) which were active with Rh_2 cells were demonstrated in antiserums containing agglutinins (anti-Rh′) for a different Rh subtype (Rh_1). This observation suggests further study on the specificity and origin of blocking antibodies in respect to subtypes of Rh agglutinogen.*

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* Since the completion of this study our attention has been called to a publication by Race[3] in which blocking antibodies of standard anti-Rh specificity were demonstrated in anti-Rh′ serums. This observation was also made by Wiener[4] whose findings are awaiting publication.

[3] Race, R. R., *Nature*, 1944, **153**, 771.

[4] Wiener, A. S., *Science*, in press.

14847 P

The Toxic Effect of Influenza Virus in the Rabbit Eye.*

C. A. Evans and E. R. Rickard. (Introduced by R. G. Green.)

From the Department of Bacteriology and Immunology, University of Minnesota, and the Influenza Laboratory, Minnesota Department of Health.

Injection of type A and B influenza virus into the rabbit eye has been found to cause corneal opacity without multiplication of the virus.

Materials and Methods. The PR8 strain of influenza type A and the Lee strain of influenza type B virus were used in the form of allantoic fluid collected from infected chick embryos 48 hours after inoculation and preserved by freezing on CO_2 ice. The 50% mortality[1] titer of the PR8[2] strain in mice was $10^{-5.5}$ and the 50% infectivity titer in eggs[3] was $10^{-8.0}$ The corresponding titers for the Lee[4] strain were $10^{-3.7}$ in mice and $10^{-7.8}$ in eggs. Specific antisera were obtained from ferrets 10 days after intranasal inoculation with either the PR8 or the Lee strain of virus.

Young adult domestic rabbits, in nearly all instances of the albino variety, were inoculated under ether anesthesia by insertion of a 26-gauge needle through the cornea near the limbus. After withdrawal of slightly more than 0.2 cc of aqueous humor, approximately 0.2 cc of the inoculum was injected.

Observations. In the course of several experiments 15 rabbit eyes were inoculated with the PR8 virus and 17 with the Lee virus as described. Without exception there developed a haziness of the cornea which progressed to a complete opacity. As a rule the cornea remained clear for 48 hours, except for the transient alteration due to trauma at the point of inoculation. The reaction was definite on the third day and attained a maximum on the fourth or fifth day, at which time the cornea was thickened and obviously edematous. In a few instances, the reaction was more rapid, some haziness being apparent at 24 hours and the opacity being complete at 72 hours. With the exception of moderate conjunctival injection and congestion of the iris, lesions were not noted in other parts of the eye than the cornea.

In 10 eyes inoculated with normal allantoic fluid and 4 with mixtures of normal allantoic fluid and normal or immune ferret sera no lesions were observed. Infected allantoic fluid inactivated by formalin 1:1000 for 12 hours or by temperature of 56°C for five minutes did not produce lesions. Likewise Seitz filtrates of infected fluids caused no pathological changes.

Lesions were only produced by the inoculation of large amounts of active virus. When the inocula were diluted 10 times in horse serum-broth typical opacity resulted; how-

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* This work was supported by funds from the International Health Division of the Rockefeller Foundation, the John and Mary R. Markle Foundation, and the Graduate School of the University of Minnesota.

[1] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **38**, 59.

[2] Francis, T., Jr., *Science*, 1934, **80**, 457.

[3] Hirst, G. K., *J. Immunol.*, 1942, **48**, 285.

[4] Francis, T., Jr., *Science*, 1940, **92**, 405.

ever, on diluting the inocula 100 times the eyes remained normal.

Neutralization tests were carried out by injecting a mixture of equal parts of undiluted serum and virus into the rabbit eye. Homologous antiserum from convalescent ferrets afforded complete protection. Normal ferret serum and heterologous influenza antiserum, type A and B, failed to protect the eye.

Tests for the persistence or multiplication of virus in the eye were carried out at intervals ranging from 8 hours to 4 days after inoculation. Aqueous humor or a suspension of tissues from the anterior portion of the eye were injected into 6-11-day-old chick embryos in each test. The results of two experiments showed that the virus disappeared quickly from the aqueous humor. Tissues from the anterior portion of the eye retained small amounts of virus (end points 10^{-2} or 10^{-3}) for three days. On the fourth day the virus was practically absent as only one out of 12 eggs injected with a 10^{-1} dilution of ocular tissues was infected.

Discussion. Inasmuch as there appeared to be a complete lack of multiplication of influenza virus in the eyes of rabbits, the severe corneal damage was attributed to a toxic effect and not to infection. The type specificity of the neutralizing power of antiserum shows that the toxic substance is not a nonspecific irritant in the inoculum but must be either the virus particle itself or some other antigenic substance generated by the virus in the infected ferrets as well as in chick embryos.

The recent reports of Rake and Jones[5] have shown a toxic effect of the viruses of lymphogranuloma venereum, mouse pneumonitis, and meningo-pneumonitis. It would appear that the corneal opacity observed in these experiments and neurologic disturbances observed by the Henles in mice[6] are probably caused by toxic properties of the influenza viruses.

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[5] Rake, G., and Jones, H. T., *J. Exp. Med.*, 1944, **79**, 463.

[6] Henle, G., and Henle, H., *Science*, 1944, **100**, 410.

14848

Prolongation of the Action of Penicillin After Intramuscular Injection.*

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Penicillin is so rapidly excreted from the body after parenteral injection that frequent administrations are necessary to maintain it continuously in the blood in measurable amounts. Although intermittent therapy may often be adequate, particularly when this involves spikes of high penicillin concentrations immediately after the injections, it is probable that a long high plateau is still more desirable. To this end, several procedures have been devised to delay the excretion, or retard the absorption, of penicillin after injection.

Rammelkamp and Bradley[1] and Beyer and co-workers,[2] using diodrast or para-aminohippuric acid to interfere with renal function, succeeded in depressing the excretion of penicillin in the urine of men and dogs. So far, this rather unphysiological method has apparently not received adequate clinical trial.

Raiziss[3] found that, when penicillin was injected intramuscularly into rabbits as a suspension in a vegetable oil, the blood remained bacteriostatic considerably longer

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* Supported by the Rockefeller Fluid Research Fund and by the Winthrop Fellowship in Pharmacology.

[1] Rammelkamp, C. H., and Bradley, S. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

[2] Beyer, K. H., Woodward, R., Peters, L., Verwey, W. F., and Mattis, P. A., *Science*, 1944, **100**, 107.

[3] Raiziss, G. W., *Science*, 1944, **100**, 412.

TABLE I.
Duration of Penicillin in Blood of Dogs After Intramuscular Injection.

No. of trials	Vehicle for penicillin	Average duration measurable penicillin in the blood, in minutes
5	Physiological salt solution in distilled water	30
1	Epinephrine 2 mg in physiological salt solution	0
1	Methyl cellulose	15
1	Human plasma	15
1	Sucrose 12%	15
1	Lactose 12%	15
3	Sesame or peanut oil	25
2	Pitressin 1 cc subcutaneously, penicillin in dextrose 5%	30
2	Sorbitol 4½ to 25%	35
3	Gelatin 8.5%	40
2	Congo red 0.5 to 1% in dextrose 5%	51
1	Gelatin 8.5% with urea 5%	60
4	Lecithin 2 to 7%	67
11	Dextrose 3 to 10%	68
4	Epinephrine 2 mg in dextrose, gelatin 8.5%, or gelatin 8.5% with urea 5%	75

than when it was injected in water, and also, that such suspensions were slow to deteriorate and lose potency. Trumper and Bradley[4] delayed the absorption of penicillin after intramuscular administration by placing an ice-pack over the area of injection to limit the local circulation. Clinical results in gonococcal infections appeared to be satisfactory, but the method was somewhat cumbersome and unpleasant. Finally, Romansky and Rittman[5,6] used suspensions of penicillin in peanut oil containing from 1 to 5% beeswax. While this method may be criticized on the basis of the residue of oil and wax which will be left at the site of injection, the foreign accumulation apparently is not harmful.

In a survey of the problem, various agents were tried by us, in dogs and in man, in an attempt to obtain a more satisfactory prolongation of the residence of penicillin in the body.

Methods. Various solutions or suspensions of sodium or calcium penicillin were prepared, given intramuscularly to dogs or humans, and blood samples taken at appropriate times thereafter. Estimations of penicillin in the blood were made by means of the method of Wolohan and Cutting.[7]

Results in Dogs. Penicillin was given intramuscularly to 11 dogs on 48 occasions, in amounts of 1000 to 4000 units. The dogs weighed from 7.7 to 18 kg, but 7 weighed about 10 kg. The variations in penicillin dosage, and in the weights of the dogs appeared to have little effect on the conclusions reached, perhaps because several different agents were tried in each dog. Likewise, no differences were noticed on 3 tests in which calcium penicillin was substituted for sodium penicillin. The total volume of solution injected was 1 or 2 cc. The lowest concentration of penicillin measured was 0.01 unit per cc.

The results as seen in Table I, showed that water, vegetable oils, methyl cellulose, human plasma, sucrose, lactose, sorbitol, gelatin, and pitressin did not substantially prolong the stay of penicillin in the body. On the other hand, epinephrine, congo red or lecithin in dextrose, gelatin with urea, and dextrose significantly prolonged the time over which penicillin could be demonstrated in the blood. None of these latter agents, with the possible exception of epinephrine, was more effective than dextrose alone. There was no essential difference in the height of the penicillin level in the blood 15 minutes after injection with the different vehicles.

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[4] Trumper, M., and Hutter, A. M., *Science*, 1944, **100**, 432.

[5] Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

[6] Romansky, M. J., and Rittman, G. E., *Bull. U. S. Army Med. Dept.*, No. 81, 1944, 43.

[7] Wolohan, M., and Cutting, W., *J. Lab. Clin. Med.*, in press.

TABLE II.
Duration of Penicillin in Blood of Humans After Intramuscular Injection.

No. of trials	Dose of penicillin in units	Vehicle for penicillin	Average duration of measurable penicillin in the blood, in hours
11	80,000	Physiological salt solution	2.5
11	”	5% dextrose	3.5
4	”	5% dextrose plus 0.25 to 0.5 mg neosynephrine hydrochloride	4.0
1	40,000	Physiological salt solution	3.0
1	”	5% dextrose	4.0
1	25,000	5% dextrose	2.0
1	”	5% dextrose plus 0.25 mg neosynephrine hydrochloride	4.0
1	20,000	Physiological salt solution	1.0
2	”	5% dextrose	1.1
2	”	5% dextrose plus 0.25 mg neosynephrine hydrochloride	1.0
2	15,000	Physiological salt solution	1.25
2	”	5% dextrose	1.5

Results in Man. On the basis of the trials in dogs, penicillin was administered to patients and normal humans in dextrose alone, and in a combination of dextrose with a vasoconstrictor. Thirty-nine sets of observations were made on 12 patients. The concentration of penicillin was 5000 units per cc.

In nearly all cases, as shown in Table II, dextrose as a vehicle extended the period over which penicillin could be measured in the blood. With the smaller doses this extension could hardly be called important, but with large doses it is great enough to be therapeutically valuable. The addition of neosynephrine to the solution prolonged the active penicillin concentration still further, but probably not enough to justify the slight hazard involved. One of the patients had a symptomless rise in blood pressure from normal to a systolic pressure of 180 mm of mercury.

Discussion. Penicillin was administered to dogs and humans intramuscularly in a variety of vehicles in an attempt to prolong its sojourn in the body. Only dextrose, of the agents tried, appeared to furnish a satisfactory and harmless extension of its action. No explanation is offered for this effect, but it appears to be substantial enough to be of clinical value when large doses of penicillin are used. Unlike the low blood concentrations produced by mixtures of penicillin with peanut oil and beeswax, a high concentration of penicillin is present in the blood immediately after injection, and no foreign substance remains in the tissues. However, it may be that a long-continued low concentration is more effective therapeutically than separated spikes of higher concentration, as suggested by the work of Romansky and Rittman.[5,6] When such mixtures are not chosen, and at present their use is limited, there seems no doubt that the penicillin should be dissolved in dextrose, and not in physiological salt solution.

Conclusion. Penicillin is demonstrable in the blood after intramuscular injection significantly longer when the vehicle is 5% dextrose instead of the usual physiological salt solution.

We are indebted to Mrs. Mary Wolohan for technical assistance.

The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

14849

Certain Properties of Theiler's Virus, Especially in Relation to Its Use as Model for Poliomyelitis.

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Theiler[1] and the writer[2] have stated that in view of the close resemblance between the properties of Theiler's virus and that of human poliomyelitis, and the similarity of certain clinical, pathological, and epidemiological features of Theiler's disease (spontaneous mouse encephalomyelitis; mouse poliomyelitis) with human poliomyelitis, the murine virus and malady might be employed as a model for problems of the human disease. This is especially true since the former is a natural disease, arising spontaneously in mice, and not an experimentally induced infection, as is the case when the virus of human poliomyelitis is transmitted to monkeys. Theiler based his opinion on the reactions of the first 5 viruses (later called "Strain 1") which he isolated first in 1933,[3] from the central nervous system of a mouse with spontaneous encephalomyelitis. These viruses he designated as "strains of low virulence"; hereafter in this paper they will be referred to as TO virus (*i.e.*, Theiler's original virus). The writer,[4] on the other hand, affirmed the similarity between the murine and human viruses, and the diseases induced by them, from his experiences chiefly with TO virus isolated in 1939 from the intestinal contents of normal stock mice.[4]

Theiler and Gard[5] later recovered by accident two highly virulent "strains," the GD VII and the FA, from mice that became ill during experiments with yellow fever virus. They were characterized by producing shorter incubation periods and higher mortality rates; they showed greater invasiveness by peripheral routes (intranasal; intraäbdominal) of inoculation but were immunologically related to, though not identical with each other and the earlier TO virus. Although the GD VII, FA, and TO viruses had certain features in common, *e.g.*, size of their active particles, the reactions of the first two in animals were so different from the TO virus as to raise the question of whether they could serve as models for the study of human poliomyelitis.

The present paper will concern itself with (a) certain properties of Theiler's virus to supplement others already reported and thus to enhance its value as a model for research on poliomyelitis problems; (b) the selection of the strain that represents best this function of the virus.

Susceptibility of Mouse Strains. Webster,[6] by inbreeding from a hybrid stock of albino mice, developed a W-Swiss strain particularly susceptible to louping-ill and St. Louis encephalitis viruses, and more recently Casals and Schneider[7] found this stock highly responsive to Russian tick-borne encephalitis. The Rockefeller Institute (R.I.) strain of mice, on the other hand, are resistant to certain neurotropic viruses to a degree which prevents their experimental use. Although other viruses (rabies,[6] lymphocytic choriomeningitis,[8] and vesicular stomatitis[9]) have been shown to be uniformly infective in both varieties of mice, investigators regard the Swiss mouse as especially susceptible to neurotropic viruses in general; at least, no instance has been hitherto noted in which a response to a virus was

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* With the technical assistance of Betty Lee Wilde and Mary Halsey.

[1] Theiler, M., *Medicine*, 1941, **20**, 443.

[2] Olitsky, P. K., *J. Exp. Med.*, 1940, **72**, 113.

[3] Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; *Science*, 1934, **80**, 122.

[4] Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 434.

[5] Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

[6] Webster, L. T., *J. Exp. Med.*, 1937, **65**, 261.

[7] Casals, J., and Schneider, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 201.

[8] Casals, J., unpublished data.

[9] Olitsky, P. K., Sabin, A. B., and Cox, H. R., *J. Exp. Med.*, 1936, **64**, 723.

TABLE I.

Comparison of Response of One-Month-Old Swiss and Rockefeller Institute Strains of Mice to Injection of Theiler's Virus.

Strain of mouse	Virus	Dose	Route	No. used	Results No. dead or with signs	Survivors
R.I.	GD-VII	10^{-1}	iper.	25	1 D-3;[*] 5 D-4; 4 D-5; 2 Pr-5;[†] 1 Ci-6; 1 D-7; 2 D-8; 1 D-15; 1 D-16	7 normal[‡]
W-Swiss	”	10^{-1}	”	25	2 D-8; 1 D-8; 1 D-9	21 normal
R.I.	”	10^{-2}	icer.	15	1 Co-1; 5 D-2; 1 Pr-2; 2 Co-2; 6 W-2	0
W-Swiss	”	10^{-2}	”	15	1 D-1; 3 D-2; 1 Pr-2; 2 Co-2; 8 W-2	0
R.I.	”	10^{-6}	”	15	3 D-3; 1 LAP-3; 4 D-4; 2 Co-4; 5 W-4	0
W-Swiss	”	10^{-6}	”	15	2 D-3; 1 AP-3; 1 LAP-3; 1 RAP-3; 1 W-3; 2 D-4; 1 Co-4; 1 W-4; 2 D-5; 1 D-6	2 normal
R.I.	TO	10^{-1}	iper.	12	1 RPP	1 paralyzed 11 normal
W-Swiss	”	10^{-1}	”	12		12 normal
R.I.	”	10^{-2}	icer.	20	2 APP-6 (D8);[§] 2 D-7; 1 PP-7 (D8); 2 AP-7 (D9); 1 AP-7 (D10); 2 APP-7 (D-13); 1 AP-8 (D9); 1 PP-9 (D13); 1 AP-10 (D11); 1 AP-10 (D22); 2 AP-10; 1 D-13; 1 AP-13 (D15); 2 APP-13	4 paralyzed
W-Swiss	”	10^{-2}	”	20	1 AP-6 (D13); 3 AP-8 (D13-14); 1 PP-9 (D15); 1 AP-10 (D16); 2 APP-11 (D17-18); 2 APP-11; 6 APP-13; 2 PP-14	10 paralyzed 2 normal

[*] 1 D-3—1 mouse found dead on 3rd day after inoculation.

[†] 2 Pr-5 = 2 mice prostrate on 5th day; Ci = circling; Co = convulsive; W = generalized weakness.

[‡] Normal = not at all affected.

[§] 2 APP-6 (D8) = 2 mice, anterior limbs partially paralyzed on 6th day, died on 8th day. 1 PP = 1 mouse, posterior limb paralysis; AP, anterior limb paralysis; L = left and R = right. In the GD VII strain, signs were preagonal, the mice dying in a few hours after their first appearance.

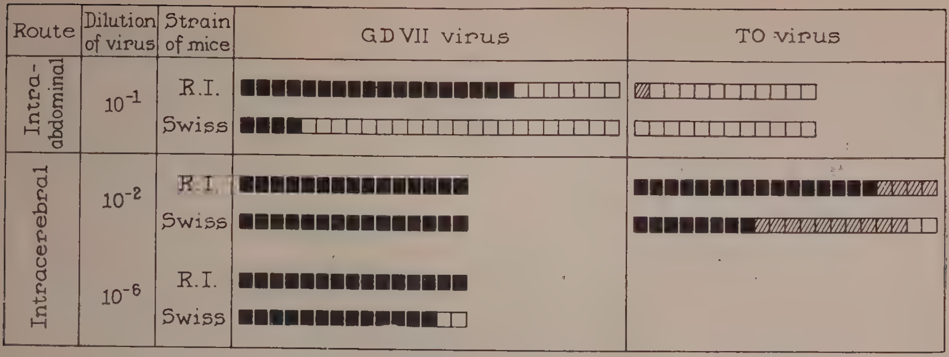
greater in the R.I. stocks of mice than in the Swiss. How this applies to the strains of Theiler's virus will be revealed from a typical experiment shown in Table I which presents details, and its graph.

It is clear that both the GD VII and TO active agents are more "virulent" in the Institute than in the Swiss variety of albino mice, as manifested by higher incidence and mortality rates and smaller number of normal or paralyzed survivors among the Institute stock. The effects follow central (CNS) introduction of TO and peripheral (intraäbdominal) injection of GD VII virus.

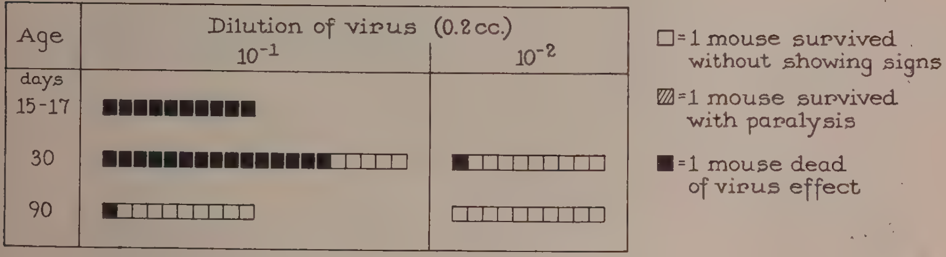
The finding that the W-Swiss mice are less susceptible to Theiler's virus than the R.I. is unexpected since it is a reversal of the hitherto accepted greater responsiveness of the Swiss mice to certain neurotropic viruses in general. The question now is, What relationship exists between selective susceptibility and the virus carrier state of normal animals?[4] Both normal stocks have been found to harbor TO Theiler's virus in the intestinal tract.[4,5] In tests performed concomitantly with the present series, the R.I. and W-Swiss mice, kept in separate animal houses, were again positive. The quantitative difference in individual incidence of carrier state and in amount of virus harbored by each carrier between the two strains of mice is yet to be determined. Care should therefore be taken to avoid discrepancies in results by selecting only single stocks of mice and not mixing strains in the same test.

The results were observed in mice of the same age,[†] one month old, for, as will be discussed in the next section, age is a factor determining natural susceptibility to Theiler's virus.

[†] Age of animals is commonly used in comparative tests, such as the present ones, rather than weight. The weight of the W-Swiss mice of this study averaged 11.7 g and of the R.I., 17.4 g at one month of age. At all comparable ages the weight of the former is considerably below that of the R.I. mice; the latter are never overtaken in weight by the W-Swiss.



Graph of Table I



Graph of Table II

FIG. 1.

TABLE II.

Influence of Age on Intraäbdominal Inoculation of GD VII Strain of Theiler's Virus in R. I. Stock Mice.

Age (days)	Dose (0.2 cc)	No. used	Results No. dead or with signs	Survivors
90	10-1	10	1 LAP-7 (D7)	9 normal
90	10-2	10	None reacted	10 ,,
30	10-1	20	2 D-4; 2 Pr-4 (D4-5); 4 D-5; 1 Ci-5 (D 5); 1 D-6; 1 D-7; 1 LPP-7 (D8); 2 D-8; 1 D-9	5 ,,
30	10-2	10	1 D-7	9 ,,
15-17	10-1	10	7 D-3; 1 Ci-4 (D4); 1 D-7; 1 D-10	0

Age Factor. Theiler[3] has previously demonstrated the influence of age on resistance to TO virus injected intracerebrally and intranasally; the resistance develops rapidly in Swiss mice from birth to 6 or 7 weeks of age. The following results (Table II) were obtained when the more susceptible R.I. mice were given GD VII material intraäbdominally. The resistance shown by various strains of mice of differing age against peripheral administration of the TO Theiler's virus has already been reported by others[3,10,11] and the writer[2] (see also Table I).

It is thus clear that the degree of invasiveness of GD VII virus from a peripheral site, in relation to age of the host, corresponds more generally with that of the encephalitis viruses,[12] while the resistance shown against the TO virus given by such routes compares with that of the rodent strains (Lansing type) of polio-

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[10] Gildemeister, E., and Ahlfeld, I., *Centr. Bakt.*, I Abt., *Orig.*, 1938, **142**, 144.

[11] Iguchi, M., *Kitasato Arch. Exp. Med.*, 1939, **16**, 56.

[12] Lennette, E. H., and Koprowski, H., J. *Immunol.*, 1944, **49**, 175.

myelitis virus and with other neurotropic viruses such as vesicular stomatitis in mice.[13]

Signs of Infection with GD VII Virus. Whether the GD VII virus is introduced into the CNS or intraäbdominally, its effects have been reported to be different from the TO virus in that most animals succumbed promptly after showing signs—the course of the disease was therefore shorter, as was also the incubation period, and the first sign was often hyperexcitability followed by spasmodic or convulsive movements.[5]

In the present study it was found that after intracerebral or intraäbdominal injection of GD VII virus, the incubation period in young mice (15 to 17 days old) is usually 3 days, and in older ones (one month or older), from 4 to 9 days. The signs are referable to meningeal and encephalitic involvement: hyperexcitability, circling, spasmodic and convulsive movements, and only occasionally weakness or paralysis of one or more limbs. Signs invariably precede death by a few hours. In most instances mice are found dead before any disturbance is noticeable. The signs here are thus different from the poliomyelitis-like syndrome which develops after TO virus is inoculated: the longer incubation period; the development of flaccid paralysis of various portions of the limbs, the rapidly developing muscular atrophy; the deformities; the general well-being of the host during most of the course of the disease, and the survival, through good nursing, of the majority of the paralyzed.

Pathology. An examination of several thousand sections of the CNS of mice which had succumbed to GD VII and to TO virus reveals that in the former the lesions are referable to a meningoencephalitis comparable to that produced in the mouse by the so-called virus encephalitides, such as Russian tick-borne, St. Louis, Japanese B, and West Nile. The chief features are massing of leucocytes and plasma cells in the meninges, in the choroid plexus and ependyma, and of leucocytes and glial cells about blood vessels, the walls of certain ones showing degeneration and, beyond, occasional perivascular hemorrhage. Degeneration and necrosis of neurons predominate and are present throughout the brain; areas of necrosis and gliosis and hemorrhage are also seen. Important to record is that the spinal cord is, as a rule, free from any noteworthy lesions. This picture is in sharp contrast to that found in mice that succumbed to TO virus infection, as previously described.[14] Here the changes induced are present caudally from the level of the thalamus through the spinal cord. In the brain neuronal degeneration and necrosis, along with neuronophagia, are not as conspicuous as the mesodermal-glial lesions but this distribution is reversed in the spinal cord, where neuronal damage is outstanding. From the viewpoint of pathology, GD VII-virus infection is seen as a meningoencephalitis, TO as an encephalomyelitis or a poliomyelitis.

Serum-Virus Neutralization Tests. Theiler and Gard[5] and Theiler[1] have already demonstrated the insusceptibility of mice, infected first with TO virus, to GD VII virus given intracerebrally but the resistance developed was shown to be relative, not uniform or complete: the two were not found to be immunologically identical. Moreover, the possibility was mentioned[5] that the resistance may have been only a reflection of the "interference" phenomenon.

In serum-virus neutralization tests by the intracerebral method in R.I. mice,[‡] a certain relationship, but not identity, between TO and GD VII viruses could be affirmed. Thus a serum deriving from rabbits repeatedly injected with active GD VII virus neutralized 100 LD_{50} of the homologous virus. When mixed with TO virus and incubated for one hour at room temperature, it prolonged the incubation period (*e.g.*, with normal monkey serum plus 10^{-3} virus dilution the period averaged 12 days, as against 19 when the rabbit antisera were used). Little or no delay was observed when antiserum to TO virus, deriving from convalescent cotton rats, was added to GD VII virus. Protection from disease by means of both antisera could not be shown by statistical analysis.

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[13] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1937, **66**, 15, 35; 1938, **67**, 201.

[14] Olitsky, P. K., and Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 79.

[‡] Thanks are due Dr. Max Theiler for his cooperation and generous gift of anti-TO and anti-GD VII sera, and to Dr. I. M. Morgan for performing these tests, reported here with permission.

Discussion. The term "strain" as applied to GD VII virus when it was first recovered, and later,[5] was not intended to indicate biological taxonomy, that is, as representing descendants from a common stock; it was so called for convenience only, meaning sample of virus isolated. Indeed, the malady from which it was recovered was not clearly one of the original Theiler's disease.[5] It is suggested that it be designated henceforth as Theiler GD VII virus. But this question of classification is not particularly important; what is significant is that the GD VII virus and the disease it induces have features not in common with the original TO Theiler's virus and its disease. The characteristics as here described, when added to others already recorded,[5] such as activity in high dilution (10^{-7}) and greater invasiveness into the host's tissues constitute elements of a picture not that of mouse poliomyelitis—natural or experimental—or its virus.

Conclusion. Additional properties of Theiler's virus—both the original and the GD VII—are described. The GD VII virus§ has been found to differ in several characteristics from the original Theiler's virus; the differences are great enough to make it unusable as a "model" for the study of human poliomyelitis.

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§ What applies to the GD VII virus may or may not apply to the FA virus which was not available for study (*Cf.* reference 5).

14850

Comparative Effects of Thromboplastin, Lecithin, and Saline on Mortality of Mice Following Experimental Burn Shock.*

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Recent investigations on the systemic chemotherapy of shock resulting from standardized experimental burns have disclosed that isotonic saline solution[1,2] and a fraction from commercial liver extract[2] will significantly increase the mean survival time of groups of treated mice over control groups.

The purpose of this report is to present evidence for the existence of a principle in a commercial cattle brain extract (Thromboplastin, USP) which is effective in prolonging the survival time and in decreasing the mortality of mice which have been subjected to experimental burns.

Method. White male mice ranging in weight from 20 to 25 g were divided, at random, into 3 groups each of which was injected intraperitoneally (approximately one-half hour prior to the application of trauma) in doses of 0.8 cc per 100 g as follows: (1) saline, 0.9% NaCl solution; (2) lecithin, 1% suspension made by homogenizing soybean lecithin with physiological saline solution in a Waring blendor; and (3) thromboplastin (Local-Cutter) diluted to a 1% homogeneous suspension with isotonic saline.

The mice were fasted 12 hours preceding the experiment, and neither food nor water was given once the test was started. This was done in order to minimize the number of variables since nutrition might conceivably be a factor and administration of food or water might influence survival time. A uniform degree of trauma in the mice was attained by immersing each etherized animal completely up to the

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* Part of the experiments were carried out at the University of California through the courtesy of Dr. Ernest W. Page.

[1] Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1943, **58**, 513.

[2] Prinzmetal, M., Hechter, O., Margoles, C., and Feigen, G., *J. Am. Med. Assn.*, 1943, **122**, 720.

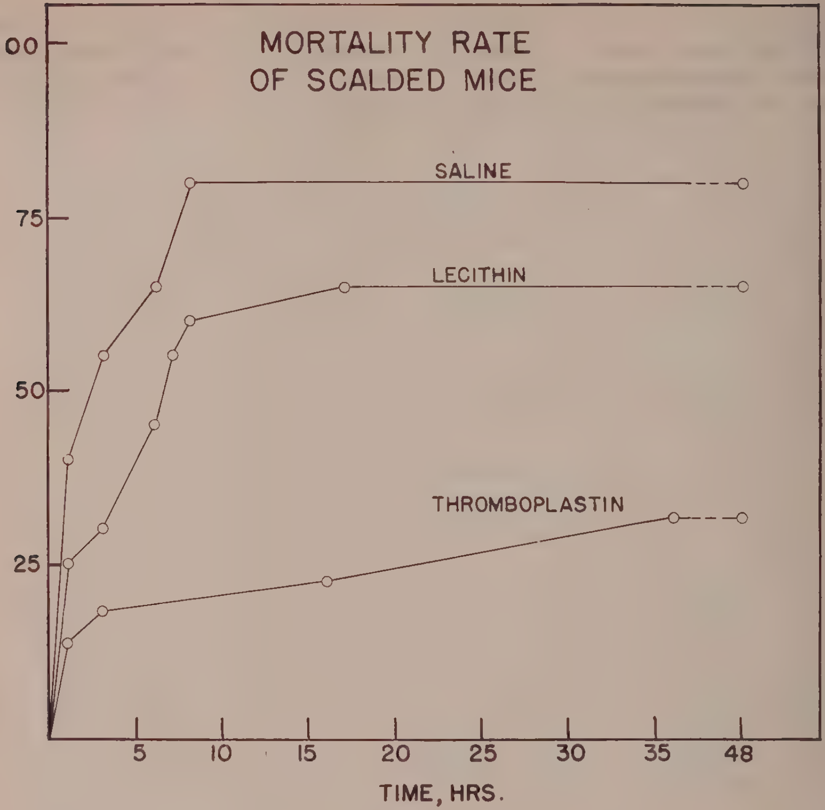

FIG. 1.

region of its neck in water thermostatically controlled at 60 ± 0.25°C for a period of 15 seconds. The animal, upon being withdrawn from the water bath, was laid gently on cotton batting and "blotted" of any excess water. At the conclusion of the scalding, every group was placed in a separate cage for observation and the time of death of each mouse was recorded.

A short time following their withdrawal from the hot water bath, the animals became dyspneic, exhibited a decrease in peripheral circulation, as evidenced by a decline in skin temperature, became stuporous and finally died. Postmortem changes regularly included profound visceral and pulmonary congestion. Hemorrhagic adrenals and hematuria were frequently observed.

The experiment was terminated at the end of a 48-hour period, because mice surviving beyond this time would be dying no longer from the effects of the scald, but would be succumbing to infections and other morbid changes.

Results. Fig. 1 shows the time-mortality characteristics for each of the 3 groups of mice, based upon a 48-hour experimental period. Each observed point represents the ratio of a number of mice dead at that point to the total number of mice used in the entire group.

Comparison of times for 50% mortality brings to light the following differences among the 3 groups: Group 1, about 3 hours; Group 2, between 6 and 7 hours; for Group 3, a 50% mortality was never reached within the 48-hour period.

Statistical analyses, based on the proportion of decedents to survivors, were carried out by

TABLE I.
Statistical Analysis of Data Based on χ^2

Group	Mice				Avg survival time in hr*	Chi² (χ^2)	''P''†
	Lived	Died	Total	% survived			
1	4	16	20	20	11.8 ± 4.3		
3	15	7	22	68	36.9 ± 4.2		
Total	19	23	42			9.92	<0.01
1	4	16	20	20	11.8 ± 4.3		
2	7	13	20	35	19.6 ± 5.0		
Total	11	29	40			1.13	<0.30

Group 1 received saline, Group 2, lecithin, and Group 3, thromboplastin.
* Including a standard error of the mean calculated as follows:

$$\frac{\sqrt{\frac{\Sigma d^2}{n}}}{\sqrt{n}}$$

where ''d'' is the deviation and ''n'' is the number of cases minus one.
† Values below 0.05 are considered significant.

the method of R. A. Fisher.[3] The degree of independence of one group from another is expressed by chi² (χ^2) for a given number of degrees of freedom; in this case, one. Survivors are those mice which were alive at the expiration of 48 hours. P is the probability of this value of chi² occurring by chance for one degree of freedom.

Treatment of the data in this manner shows that although lecithin does not differ significantly from saline in protecting the animals, there is an unmistakable superiority of thromboplastin over saline in saving lives of animals to a 48-hour level.

Discussion. Shock, as the result of burns, is characterized by the increased transudation of fluid and serum proteins from the vascular to the extravascular compartment due to changes in the permeability of the capillary endothelium. The increase in capillary permeability is generally ascribed to the action of some "toxic factor" which is elaborated from the products of tissue destruction at the site of trauma. The presence in the circulation of such a factor is presumably responsible for much of the visceral damage found at autopsy following death from burns. The organs most constantly damaged are those receiving the greatest blood supply; namely, the liver, spleen, kidneys, lungs, and members of the reticulo-endothelial system generally. Liver damage, usually characterized by central necrosis and fatty infiltration is considered an important cause of death after a severe burn.

Evidence has been presented by several investigators[4-9] that regardless of initiating factors, a prominent finding in shock is the decrease in blood platelet concentration and the increase in the coagulation time of the blood. This decreased coagulability of the blood has been ascribed by Quick[4] to the production of an anticoagulant which resembles heparin in its action, though Eagle's finding[5] that addition of platelets to shock blood would not cause clotting indicates that Quick might have been dealing with an antithrombin rather than an antiprothrombin.

The failure of lecithin to be effective as thromboplastin argues that the action of thromboplastin is a specific one. The thromboplastin, although relatively crude, was prepared for its cephalin content. Thus, the effect of thromboplastin is due either to the action of cephalin or to some other substance present in the rather crude extract. Since the two phospholipids are closely allied structurally, the possibility of a caloric influence is eliminated, for these substances are virtually isodynamic and any other material contained in the crude extract which might have this effect would be so low in its concentration as to be negligible from this standpoint.

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[3] Fisher, R. A., *Statistical Methods for Research Workers,* 1936 Ed., Oliver and Boyd, London.

[4] Quick, A. J., *Am. J. Physiol.*, 1936, **116**, 535.

[5] Eagle, H., Johnston, C. G., and Ravdin, I. S., *Bull. J. Hopk. Hosp.*, 1937, **60**, 428.

[6] Kopeloff, N., and Kopeloff, L. M., *J. Immunol.*, 1941, **40**, 471; 1939, **36**, 83, 101.

[7] Kinsell, L. W., Kopeloff, L. M., Zwemer, R. L., and Kopeloff, N., *J. Immunol.*, 1941, **42**, 35.

[8] Schultz, E. W., Proc. Soc. Exp. Biol. and Med., 1924, **22**, 343.

[9] Krueger, A. P., and Schultz, E. W., Proc. Soc. Exp. Biol. and Med., 1925, **23**, 153.

Data on the mechanism of action must await purification of the active principle. However, the experiments of Glenn, Peterson, and Drinker[10] recently reviewed by Cope[11] prove that thromboplastin is absent in burn edema fluid. In the light of our results this fact would indicate that the function of the injected thromboplastin is to augment the supply of this substance and thus to prevent excessive transudation and fluid loss. An experiment performed to determine whether the material was effective if administered after shock had ensued yielded equivocal conclusions. The animals treated with thromboplastin seemed to die faster than saline-treated animals, indicating that the material was toxic to shocked mice. The crude saline extract of cattle brain contains substances which are inactive as far as *normal* mice are concerned, but such compounds as histamine, which are present in these preparations, will become markedly harmful if they are administered at a time when the animal's normal detoxifying systems have been deranged by burn shock. Further purification is necessary to establish whether the toxicity of the crude preparation is responsible for its lack of efficacy when administered after shock has once been established, or whether some physiological intermediate present in normal animals but damaged in shocked mice, is blocked so that administration of a precursor is ineffective.

Conclusions. 1. Mice injected intraperitoneally with a 1% suspension of thromboplastin in isotonic saline solution showed a greater mean survival period than mice injected with either isotonic saline or with a 1% suspension of lecithin in isotonic saline. 2. Mice receiving thromboplastin showed a higher ultimate survival rate than those receiving either lecithin or saline. 3. Analysis by chi^2 showed a significant difference between thromboplastin and saline but not between lecithin and saline. 4. Intraperitoneal injection of thromboplastin one hour after traumatization of the animals seemed to exert a toxic effect.

The authors wish to express their appreciation to Mr. Frank Lanni for the figure.

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[10] Glenn, W. W. L., Peterson, D. K., and Drinker, C. K., *Surg.*, 1942, **12**, 685.

[11] Cope, O., *J. Am. Med. Assn.*, 1944, **125**, 536.

14851 P

Nutritional Macrocytic Anemia: Response to a Substance Other Than the Anti-Pernicious Anemia Principle.*

JANET WATSON AND W. B. CASTLE. (Introduced by Maxwell Finland.)

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Recent publications[1-7] indicate a difference

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* The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School.

[1] Napier, L. E., Das Gupta, C. R., Chaudhuri, R. N., Sen, G. N., Rai Chaudhuri, M. N., Sen Gupta, P. C., and Majumder, D. N., *Indian Med. Gaz.*, 1938, **73**, 385.

[2] Wills, L., and Evans, B. D. F., *Lancet*, 1938, **2**, 416.

[3] Davidson, L. S. P., Davis, L. J., and Innes, J., *Brit. Med. J.*, 1942, **2**, 31.

TABLE I.
Positive Hematopoietic Effect of Orally Administered Liver Extract Following Negative Effect of Parenterally Administered Liver Extract.

	Case 1		Case 2		Case 3	
Days of treatment	R.B.C. (mils)	Retics. (%)	R.B.C. (mils)	Retics. (%)	R.B.C. (mils)	Retics. (%)
	Liver Inj.—Lederle 10 U.S.P. units i.m. alternate days		Reticulogen—Lilly 1 cc and Liver Inj. (Crude)—Wilson 4 U.S.P. units i.m. daily		Special Liver Preparation* 5 cc i.m. daily	
0	1.42	0.4	3.07 (T)†	0.4	1.72 (D) (T)	0.6
2	1.03 (T)	0.4	2.56	1.0	1.57	0.0
4	1.36	0.2	2.65	0.4	1.78	0.0
6	1.21 (T)	0.2	—	0.5	1.52	0.2
8	3.50 (T)	**0.2**	—	0.9	1.28	0.2
	Liver Inj. (Crude)—Wilson 5 U.S.P. units i.m. daily				Same Preparation 51 cc 1 U.S.P. unit orally daily	
10	3.00	0.0	2.92	1.6	1.27	2.6
			Liquid Ext. Liver—Valentine‡ 1 U.S.P. unit orally daily			
12	3.26	0.2	—	1.6	1.26	8.4
14	3.37	0.0	3.02	3.2	1.46	**26.2**
16	3.15	0.1	3.45	**13.0**	1.67	16.0
18	3.50 (T)	0.0	3.65	6.2	1.97	16.6
	Liquid Ext. Liver—Valentine‡ 1 U.S.P. unit orally daily					
20	3.19	0.1	3.22	2.2	2.25	12.2
22	3.50	0.4			2.72	12.6
24	3.08	1.7			2.48	7.5
26	3.27	2.6			2.97	7.3
28	3.26 (T)	4.5	3.90	—		
30	3.46	**6.5**				
32	3.45	3.8	4.10	—		
34	3.54	4.5			3.38	3.8
36	4.10	3.8				
47 (D)	4.03	2.5				

* Liver Extract—Lilly, of which 12.75 g constitute 1 U.S.P. unit (oral), was kindly supplied by Mr. George B. Walden of Eli Lilly and Company. This material was suspended in water at pH 6.8 and sterilized by boiling. Each cc of the suspension solution contained 0.25 g of the original preparation.

† (T) = transfusion; (D) = delivery.

‡ Kindly supplied by Mr. Braxton Valentine of Valentine's Meat-Juice Company.

between the basic nutritional deficiency in Addisonian pernicious anemia and in certain macrocytic anemias usually associated with pregnancy and encountered in both the tropics and the temperate zone. Thus, in the latter no response was observed following parenterally administered liver extracts of known effectiveness in pernicious anemia but rapid blood regeneration was said to occur when liver extract, liver, or autolyzed yeast was given by mouth. Because paucity of hematological controls renders some of these interesting reports difficult of interpretation, observations were made on 3 female patients with nutritional macrocytic anemia apparently of this unusual type.

Cases 1 and 3 became anemic during pregnancy. All gave histories of striking dietary inadequacy, showed free hydrochloric acid in

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[4] Miller, H. G., and Studdert, T. C., *Lancet*, 1942, **2**, 332.

[5] Fullerton, H. W., *Brit. Med. J.*, 1943, **1**, 158.

[6] Davidson, L. S. P., Davis, L. J., and Innes, J., *Edinburgh Med. J.*, 1943, **50**, 431.

[7] Nielsen, O. P., *Acta med. Scandinav.*, 1941, **108**, 421.

the gastric contents, and had macrocytic and slightly hypochromic anemia with moderate leukopenia and thrombocytopenia. Anisocytosis and poikilocytosis of red blood cells were less marked than in equally anemic cases of pernicious anemia. The differential white counts were normal. There were no neural manifestations. The methods of dietary control and of blood counting[8] and the interpretation of daily counts of reticulocytes[9] made during successive test periods have been described elsewhere.

The hematological data summarized in Table I appear to demonstrate that prompt response to orally administered liver extracts immediately followed therapeutic failure of liver extracts given parenterally, even in multiple U.S.P. units[10] daily. Cases 1 and 2 responded to liver extract given orally after failing to respond to more refined preparations of known effectiveness in pernicious anemia given intramuscularly. This demonstrates in these patients a deficiency of some substance other than the principle effective in pernicious anemia. Case 3, unlike patients with pernicious anemia, responded to a special preparation of Liver Extract—Lilly only when it was given orally in 10 times the amount that was ineffective on intramuscular injection. This suggests that the tenfold increase in oral dosage was the cause of the therapeutic success, and eliminates as an explanation of therapeutic failure following parenteral administration a difference in chemical properties of the preparation used. The fact, however, that in pernicious anemia an aqueous solution of Liver Extract—Lilly has been found to be 60 times as active hematopoietically when injected as when given by mouth,[11] suggests an alternative explanation: Possibly the digestive organs in this type of patient form a new hematopoietic substance from the liver extract in a fashion analogous to the food-gastric factor relationship in Addisonian pernicious anemia.[8,12]

As was first pointed out for tropical macrocytic anemias,[1,2] in such exceptional patients as are here reported the basic nutritional deficiency differs from that in patients with Addisonian pernicious anemia. Consequently, the "unitarian" hypothesis concerning the etiology of pernicious and other nutritional macrocytic anemias, suggested by Strauss and Castle,[12] does not apply to these particular patients. This distinction emphasizes the desirability of a therapeutic trial of *orally administered* liver extract in macrocytic anemias, particularly those of pregnancy, when they are refractory to parenterally administered liver extracts.

We are indebted to Miss Geneva A. Daland and Miss Ruth E. Pearson for performing the blood examinations.

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[8] Castle, W. B., and Ham, T. H., *J. A. M. A.*, 1936, **107**, 1456.

[9] Minot, G. R., and Castle, W. B., *Lancet*, 1935, **2**, 319.

[10] U.S.P. Anti-Anemia Preparations Advisory Board: Third Announcement, *J. Am. Pharm. Assn.*, Practical Pharmacy Edition, 1940, **1**, 53.

[11] Strauss, M. B., Taylor, F. H. L., and Castle, W. B., *J. A. M. A.*, 1931, **97**, 313.

[12] Strauss, M. B., and Castle, W. B., *New Eng. J. Med.*, 1932, **207**, 55.

14852

Acute Toxicity of Choline Chloride Administered Orally to Rats.*

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From the recent studies on the toxicity of choline chloride in rats and mice[1,2] two pertinent facts may be reiterated: (a) The toxicity increases with increasing concentration when choline chloride solutions are given intraperitoneally to rats, (b) The toxicity following intraperitoneal administration is much higher than that following administration by stomach tube. The following experiments amplify those previously reported[1] on the oral toxicity and show that an increase of toxicity with concentration also occurred when the choline is administered by stomach tube.

Methods. 413 male and female albino rats weighing from 76 to 343 g were given 4 different concentrations of choline chloride solutions.

Groups of 5 to 30 rats each were fasted for 24 hours and then given by stomach tube various doses of each of the 4 concentrations of choline. Several doses of each of the 4 solutions administered on a single day gave fairly consistent results. A roughly linear relationship existed between dosage and percentage mortality. However, on different days marked differences were observed in the susceptibility of the rats; there was considerable scatter in the series as a whole. No explanation for this variation could be found; no correlation existed between sex or weight and toxicity, and the solutions were freshly made for each test.

Results. The results for the 2 higher concentrations (670 and 500 mg/ml) were combined and the results for the 2 lower concentrations (200 and 400 mg/ml) were combined as illustrated in Fig. 1. These values show a significant increase in mortality with increase in concentration. The LD_{50}, calculated by the method of Bliss[3] for the 2 higher concentrations was of the order of 3.4 g/kg body weight. For the 2 lower concentrations, the LD_{50} was of the order of 6.1 g/kg body weight.

The action of choline was rapid; symptoms appeared within 5 to 10 minutes. In most cases, there was an initial excitement period, characterized by jerking movements and occasional convulsions. At this time, the bloody tears (chromodacryorrhea)[4] appeared in 30 to 90% of the animals which succumbed. The excitement period was followed in a few minutes by a depression with complete relaxation and depressed respiration which finally terminated in respiratory paralysis after 15 to 30 minutes. Animals which lived longer than 30 minutes in most cases survived. A few animals which survived several hours were found dead 18 hours after the drug was given. This delayed response was not observed following the intraperitoneal administration of choline.

Summary. 1. 413 albino rats were given solutions of choline chloride by stomach tube. Four concentrations, viz., 670, 500, 400, and 200 mg/ml were used. A significant increase in the toxicity with increase in concentration was found after combining the results for the 2 higher and 2 lower concentrations.

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† The authors acknowledge the technical assistance of Raymond Kessel. Miss Elizabeth Street carried out the statistical study.

[1] Hodge, H. C., Goldstein, M. R., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 281.

[2] Hodge, H. C., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 26.

[3] Bliss, C. L., *Ann. Appl. Biol.*, 1935, **22**, 134.

[4] Tashiro, S., Badger, E., and Younker, W., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 377.

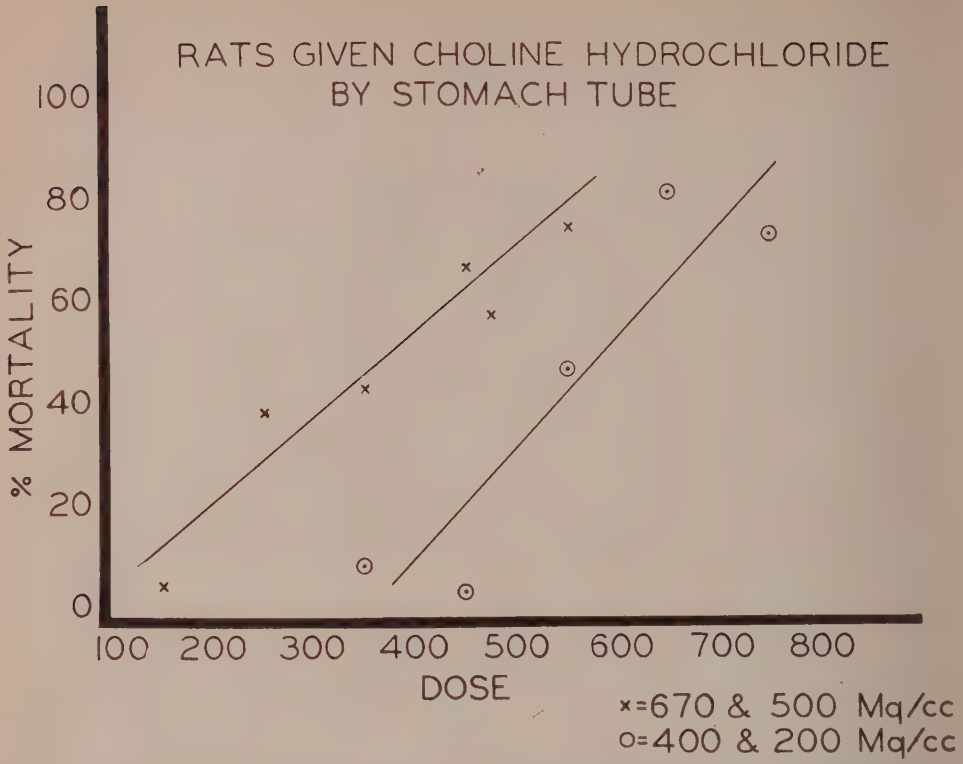

FIG. 1.

These two curves represent the average results obtained with rats given choline solutions containing 670 and 500 mg/ml and 400 and 200 mg/ml, respectively. The dose is represented as mg/100 g rat; expressed as g/kg, the abscissæ are 1.0 to 8.0, respectively.

2. The LD_{50} for the 2 higher concentrations (average 580 mg/ml) was of the order of 3.4 g/kg body weight; for the 2 lower concentrations (average 300 mg/ml) was of the order of 6.1 g/kg body weight.

14853

New Data on the Distribution of the Rh Blood Types.*

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In preceding papers,[1,2,3] family and statistical data were presented in order to test the theory of 6 allelic genes,[4] proposed to account for the hereditary transmission of the 8 Rh blood types. While the family data were in good accord with the theoretical expectations, the statistical distribution of the types in the general population differed slightly but significantly from the expected values. The principal difficulty appeared to be that an excessive number of individuals were classified as type $\mathrm{Rh}_1\mathrm{Rh}_2$, and it was suggested that the discrepancy might be due to technical difficulties.[2] As was pointed out, the reagents anti-Rh′ and anti-Rh″ used in our earlier work were not completely specific, due to the presence in the sera of traces of anti-Rh_o agglutinin. Accordingly, it was postulated that a certain number of type Rh_1 and type Rh_2 bloods had been incorrectly diagnosed as type $\mathrm{Rh}_1\mathrm{Rh}_2$. Recently, a method was devised for preparing more specific anti-Rh′ and anti-Rh″ reagents,[5,6] and the purpose of the present paper is to present our more recent findings obtained with the aid of these improved reagents.

Serological Considerations. The most frequent[7] human anti-Rh serum gives 85% positive reactions on blood samples from white individuals and parallels in specificity the standard anti-rhesus immune guinea pig sera.[8] (Such sera contain the agglutinin now designated[9] as anti-Rh_o.) This indicates that of the 3 factors,[9] Rh_o, Rh′, and Rh″, factor Rh_o is by far the most antigenic. The opportunity to produce anti-Rh′ agglutinins arises when an Rh-negative mother has an erythroblastotic infant of type Rh_1, but even in such cases usually only the agglutinin anti-Rh_o is formed. When sera containing anti-Rh′ agglutinins are obtained, they as a rule also contain a certain amount of anti-Rh_o agglutinin so that they correspond in specificity to anti-Rh'_o (anti-$\mathrm{Rh}_o\mathrm{Rh}'$) sera, which give approximately 87% positive reactions with bloods[10] from white individuals. While erythroblastotic infants of type Rh_2 are quite common, sera containing agglutinin anti-Rh″ are rare, apparently because the factor Rh″ is an even poorer antigen than Rh′. When Rh-negative women produce anti-Rh″ agglutinins, it is not surprising that their sera also contain some agglutinin anti-Rh_o, and therefore correspond to anti-Rh''_o (or anti-$\mathrm{Rh}_o\mathrm{Rh}''$), a variety of anti-Rh serum which gives slightly more than 85% positive reactions.[4,11] Obviously, sera anti-Rh'_o and anti-Rh''_o are of but little use for Rh typing, because if one wishes to determine to which of the 8 Rh types an individual belongs 3 separate reagents containing the agglutinins anti-Rh_o, anti-Rh′, and anti-Rh″ individually, rather than in combination, are required. Anti-Rh'_o and anti-Rh''_o sera are of as little value for Rh-typing as group O sera are for ordinary blood grouping.

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* Aided by a grant from the Carnegie Foundation, through the Committee of Human Heredity of the National Research Council.

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[3] Wiener, A. S., Sonn, E. B., and Belkin, R. B., *J. Immunol.*, in press.

[4] Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 316.

[5] Wiener, A. S., *Am. J. Clin. Path.*, in press.

[6] Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 63.

[7] Wiener, A. S., Davidsohn, I., and Sonn, E. B., unpublished observations.

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The opportunity to obtain sera containing the agglutinin anti-Rh′ without anti-Rh_o could arise when a type Rh_2 mother has an erythroblastotic infant of type Rh_1, because the mother in such cases shares the factor Rh_o with her child and could only react to the factor Rh′ in the child's blood. In a similar way a type Rh_1 mother with an erythroblastotic baby of type Rh_2 might produce a serum containing agglutinin anti-Rh″ alone. A case has been encountered by Levine and Waller[12] in which a type Rh_2 mother produced anti-Rh′ agglutinins without accompanying anti-Rh_o. In 2 cases seen by the authors, one referred by Dr. L. K. Diamond and the other by Dr. A. M. Young, type Rh_1 women had sera containing anti-Rh″ agglutinins of high titer and not associated with any other irregular isoantibody, so that these sera were ideal for Rh typing. Five similar cases have been observed by British investigators.[13]

A number of sera have also been obtained from Rh-negative women which seemed to contain the agglutinin anti-Rh′ alone. In view of our present knowledge that Rh_o is by far the better antigen, it would be difficult to understand how an Rh-negative mother with a type Rh_1 infant could produce anti-Rh′ agglutinins without some anti-Rh_o. More recent observations have revealed that these so-called anti-Rh′ sera are actually anti-Rh'_o sera, but containing blocking antibodies which neutralize the action of the anti-Rh_o agglutinin.[14,15] This suggested[16] the possibility of converting anti-Rh'_o serum into anti-Rh′ reagents by the simple addition of sera containing anti-Rh_o blocking antibody. With the aid of a high titered anti-Rh_o blocking serum obtained from a male patient who had had a hemolytic transfusion reaction a year previously, we have been able to test this idea and found it practicable. Anti-Rh′ and anti-Rh″ reagents prepared with anti-Rh_o blocking sera proved to be superior in specificity to reagents prepared from anti-Rh'_o and anti-Rh''_o sera in which the action of the anti-Rh_o agglutinin was eliminated by absorption or by dilution. It is with these improved reagents that the present investigation was carried out.

Before presenting our findings it should be mentioned that anti-Rh_o blocking antibodies do not inhibit the reactions of anti-Rh_o agglutinins with bloods of all Rh types to the same degree. Thus, the titer value obtained for an anti-Rh_o blocking serum is about twice as high for test cells Rh_1 as for test cells Rh_2, indicating that the Rh_o property of type Rh_2 cells is less readily blocked than the corresponding property of type Rh_1 cells. Many weak anti-Rh_o agglutinating sera also contain small amounts of anti-Rh_o blocking antibodies and this gives rise to an interesting effect, namely, that the reactions of these sera with bloods of types Rh_2 and Rh_1Rh_2 are considerably more intense than with bloods of type Rh_1. The fact that the reactions with bloods containing Rh_2 are stronger may give rise to the erroneous impression that one is dealing with an anti-Rh''_o or anti-Rh″ serum. If known blood of type Rh″ is available, it will be found, however, that the serum does not agglutinate such blood in contrast to sera which contain the agglutinin anti-Rh″. If type Rh″ blood is not available, the true nature of the serum will be revealed if a small amount of anti-Rh_o blocking serum is added. This will serve to destroy the activity of the serum, whereas in the case of true anti-Rh''_o (or anti-Rh″) sera the reactions with bloods of types Rh_2, Rh_1Rh_2, and Rh″ will hardly be affected.

Results. In Table I are presented the distribution of the Rh blood types in a series of 468 blood samples from white individuals in New York City, as compared with our previous results on a series of 1000 blood samples tested with the older and less specific reagents. While the distributions in the two series are similar, the new findings confirm the suspicion that the excess of samples diagnosed as type

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[12] Levine, P., and Waller, R. K., *Am. J. Clin. Path.*, in press. Davidsohn and Wiener have recently encountered a similar case (unpublished observations).

[13] Loutit, J. F., personal communication.

[14] Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 173.

[15] Race, R. R., *Nature*, 1944, **153**, 771.

[16] Wiener, A. S.[6]

TABLE I.

Distribution of the Rh Blood Types Among White Individuals in New York City.

Series	No. tested	Rh blood types							Gene frequencies						D = 100 − ΣRh_i*	P.E.$_D$
		Neg.	Rh_1	Rh_2	Rh_1Rh_2	Rh_o	Rh′	Rh″	*rh*	Rh_1	Rh_2	Rh_o	*Rh′*	*Rh″*		
Old series	1,000	12.9	54.1	*12.8*	*16.4*	2.6	0.9	0.3	35.9	43.4	*13.7*	3.5	1.2	0.4	+1.9	±0.5
New ”	468	14.3	54.9	15.6	12.0	2.1	1.1	—	37.8	43.1	16.0	2.7	1.4	—	−1.0	±0.9
Combined	1,468	13.3	54.4	*13.7*	*15.0*	2.4	1.0	0.2	36.5	43.3	*14.5*	3.2	1.3	0.3	+0.9	±0.5

* The symbol ΣRh_i is used to designate the sum of the gene frequencies.

Rh_1Rh_2 was due to technical difficulties. The difficulty appears to have involved the old anti-Rh′ reagent but not the anti-Rh″ reagent, so that a certain percentage of type Rh_2 bloods were erroneously classified as type Rh_1Rh_2. Since the number of technical errors in our old data appears to be relatively small, the two series have been combined, and the frequencies given for the blood types and genes in the combined series can be taken at their face value except for gene Rh_2, whose true frequency appears to be approximately 16%.

As was pointed out in previous papers,[1,2] the accuracy of the theory of 6 allelic genes can be tested by determining whether the sum of the calculated gene frequencies differs significantly from 100%. It will be seen that the new data on the distribution of the Rh types are in excellent accord with the theory, because the difference of the sum of the calculated gene frequencies from 100% is less than its probable error.

Another way of testing the theory is by making use of the observed incidence of positive and negative reactions given by the anti-Rh′ and Rh″ sera to calculate the expected frequencies of the 4 classes W, U. V, and UV, and to compare these values with the observed frequencies.

If *u*, *v*, and *w* represent the frequencies of the genes *U*, *V*, and *W* respectively, then

$$u = 1 - \sqrt{(\text{Rh}'-)}$$

$$v = 1 - \sqrt{(\text{Rh}''-)}$$

where (Rh′–) represents the percentage of negative reactions given by the anti-Rh′ serum; and (Rh″–) represents the percentage of negative reactions given by the anti-Rh″ serum.

In our new series of 468 tests, we obtained

$$(\text{Rh}'-) = 32.0\%$$
$$(\text{Rh}''-) = 72.4\%$$

Therefore,

$$u = 1 - \sqrt{0.320} = 43.4\%$$

$$v = 1 - \sqrt{0.724} = 14.9\%$$
$$w = 1 - (u + v) = 41.7\%$$

From these gene frequencies, the expected frequencies of the classes is computed as follows:

$W = w^2 = 17.4\%$ or 81.4
$U = u^2 + 2uw = 55.0\%$ or 257.4
$V = v^2 + 2vw = 14.6\%$ or 68.3
and $UV = 2uv = 13.0\%$ or 60.8

With these values may be compared the actually observed frequencies:

$W = Rh_o + \text{Neg.} = 16.4\%$ or 77
$U = Rh_1 + Rh' = 56.0\%$ or 262
$V = Rh_2 + Rh'' = 15.6\%$ or 73
$UV = Rh_1Rh_2 + Rh'Rh'' = 12.0\%$ or 56

$$\chi^2 = \Sigma \frac{(X - X_o)^2}{X_o} = 1.03$$ for one degree of freedom,† so that $P = 0.30$. Therefore, these statistical results strongly support the theory of multiple allelic genes of the heredity of the Rh blood types.

Distribution Among Negroes. Comparison of distribution of the blood groups, subgroups, and M-N types among Negroes and white individuals has revealed significant though small differences between the 2 races. Interestingly enough, the differences in distribution in the case of the Rh types have proved to be far more striking.[17] For example, the type Rh_o, which has a frequency of only 2.5% among white individuals, is 15 to 20 times as common among Negroes (42%). In recent studies on the Rh types in white individuals, rare bloods have been encountered which give reactions of intermediate intensity with one or more of the anti-sera so that they did not fit with any of the 8 standard types. These aberrant types appear to be determined by special "intermediate" genes which have increased the number of allelic genes in the Rh series to 10 or more.[18] The intermediate genes are of interest because in Negroes they are relatively common in contrast to their rarity among white individuals, and the disparity in incidence seems to be even more pronounced than for type Rh_o.

The striking differences in distribution of the Rh types among Negroes and whites are illustrated by some experiments in which bloods were tested without knowledge of their racial derivation. In one such experiment on 19 blood samples from white individuals and 2 from Negroes, the bloods of the Negroes were easily selected because one belonged to type Rh_o and the other gave intermediate reactions. This experiment was successful despite the fact that the chance of selecting the 2 bloods correctly at random was only one in 210. Naturally, other experiments were not uniformly successful because there is no qualitative difference between the Rh types of Negro and white blood, merely a difference in distribution.

There also exist pronounced differences between Negroes and whites in the distribution of the properties detected by anti-P and anti-Hr sera. As is pointed out elsewhere,[6,19] the Hr-negative and P-negative types each occur in approximately 25% of the white population in New York City, while these types each have a frequency of only 2% among Negroes. Therefore, blood specimens that are both Hr and P negative have a frequency of approximately 6¼% among whites and only 1/25 of 1% among Negroes, so that this combination is more than 150 times as common among whites as among Negroes.

Summary. New data have been compiled with the aid of improved testing sera, on the distribution of the 8 Rh blood types among white individuals in New York City. Statistical analysis of these data by the gene frequency method yielded results in satisfactory agreement with the expectations under the six-gene theory. The distribution of the Rh blood types among Negroes is strikingly different from the distribution among white individuals; the most pronounced differences involving the frequencies of type Rh_o and the so-called intermediate types. Striking differences between whites and Negroes also exist with respect to the distributions of agglutinogens P and Hr.

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† Actually the true value of χ^2 is probably even smaller because the method used here for calculating the gene frequencies is not the most efficient, though the simplest.

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14854

Cold Hemagglutinins in Visceral Leishmaniasis (Kala-Azar).

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Cold agglutinins, or autoagglutinins, have been described in a wide variety of diseases including pneumonia, pernicious anemia, leukemia, hemolytic syndromes, hepatic cirrhosis, and peripheral vascular disorders.[1] It is evident, however, that such agglutinins do not occur regularly in any of these conditions, but rather sporadically and in an exceedingly haphazard manner. On the other hand it has long been known that autoagglutinins, or cold agglutinins, are commonly present in the blood of both humans and animals suffering from trypanosomiasis,[2] while more recent observations indicate that a similar serological response occurs frequently in cases of primary atypical pneumonia.[3,4,5]

The present report deals with the discovery of cold agglutinins in the blood of 2 patients suffering from visceral leishmaniasis (kala-azar). Apparently the only reference in the literature to a similar serological observation is Manson-Bahr's brief statement that autoagglutination occurs frequently in the disease.[6] Napier says that he has seen autoagglutination in kala-azar, but does not believe it to be of common occurrence.[7]

Case No. 1—S. T., a 15-year-old Filipino school girl, was admitted to the Presbyterian Hospital on July 20, 1943. One year before admission the patient emigrated to the United States from Calcutta, India, where she had previously resided for several years. Four months before admission she first noted symptoms of an illness which was characterized by remittent chills and fever, anorexia, progressive loss of weight, aching pain in the left upper quadrant, and gradual swelling of the abdomen. On physical examination the chief findings were pallor, emaciation, and a tremendously enlarged spleen. The significant laboratory data were as follows: erythrocytes 3,600,000, hemoglobin 8.6 g, leucocytes 2500, polymorphonuclear neutrophiles 53%, lymphocytes 34%, monocytes 13%, sedimentation rate 105 mm in one hour (Westergren), Wassermann anti-complementary, serum proteins 12.8 g% (albumin 3.6, globulins 9.2), cephalin flocculation test 4+, serum cholesterol 102 mg%. Microscopical examination of the sternal marrow revealed numerous Leishman-Donovan bodies. Chemotherapy was instituted with neostibosan intravenously, and a total of 3.5 g of the drug was administered over a period of 26 days. Clinical improvement was noted within a week after treatment was begun and thereafter the temperature remained normal, the patient gained weight, and the spleen decreased rapidly in size. At the time of discharge on August 31, 1943, 37 days after admission, the patient was completely asymptomatic, and 2 months later, on October 29, the spleen could not be palpated.

Case No. 2—Th., a 23-year-old Indian seaman, was admitted to the United States Marine Hospital at Ellis Island, N.Y., on August 16, 1943.* A diagnosis of kala-azar was made and confirmed by the finding of *Leishmania donovani* in material obtained by splenic puncture. The patient was successfully treated with neostibosan. On October 5, 1943, a specimen of blood was obtained through the courtesy of Dr. Harry Most, of New York University.

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[2] Yorke, W., *Ann. Trop. Med. and Parasitol.*, 1911, **4**, 529.

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[4] Turner, J. C., Nisnewitz, S., Jackson, E. B., and Berney, R., *Lancet*, 1943, **1**, 765.

[5] Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 181.

[6] Manson-Bahr, P., *Manson's Tropical Diseases*, The Williams and Wilkins Co., Baltimore, 11th Edition, 1940, p. 191.

[7] Napier, L. E., personal communication.

* This case has been reported by Price, F. L., and Mayer, R. A., *J. A. M. A.*, 1944, **125**, 490.

TABLE I.
Titer of Cold Agglutinins for Group O Erythrocytes in Two Cases of Leishmaniasis.

		Serum dilutions								
Patient	Date	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512	1-1024
S.T.	9-10-43	++++	++++	+++	++	++	+	0	0	0
	24	++++	++++	++++	+++	+++	+	0	0	0
	10- 1	++++	++++	++++	++	++	+	0	0	0
	29	++++	++++	++++	++++	+++	++	+	0	0
	12-17	+	±	0	0	0	0	0	0	0
Th.	10- 5-43	++++	++++	++++	++++	++++	++++	++++	+++	+

Tests for cold agglutinins were performed on 5 occasions in patient S.T. and on the single specimen obtained from Th. No attempt was made to preserve a high agglutinin titer by removing the serum before the blood samples were initially cooled or chilled. However, in each instance after the clotted blood was received it was placed in an incubator at 37°C for one hour, followed by immediate centrifugation and withdrawal of the serum. Serial doubling dilutions of the serum were made in tubes containing 0.5 cc of 0.85% saline. To each tube of one series 0.5 cc of a 1.0% washed suspension of the patient's erythrocytes was added, while the tubes of the second series received 0.5 cc of a similar suspension of Group O cells. The racks were then placed in a refrigerator at 5°C overnight, and the results read immediately after removal from the cold by flipping the tubes and estimating the degree of agglutination with the naked eye. Four-plus agglutination was indicated by a tightly-packed disc of cells and one-plus agglutination by finely granular clumping. After the initial readings had been made the tubes were placed at room temperature (20-24°C) for an hour, and in each case the agglutination was observed to be completely dispelled.

The results of the tests are given in Table I and show that the sera of the patients with leishmaniasis contained cold agglutinins varying in titer from 1-128 to 1-1024. Separate data for the titers obtained with autologous and homologous erythrocytes are not given, since the values were essentially similar. Using the same technic more than 300 sera from normal subjects and from patients with a variety of other diseases were also examined, nearly all of which showed no cold agglutination whatsoever, although a small number showed weak agglutination at titers not exceeding 1-8.

Discussion. In patient S.T. the titer of cold agglutinins was found to be 1-128 on September 10, 1943, at which time the course of therapy with neostibosan had been completed and the clinical status was markedly improved, although the spleen was still very large. Seven weeks later, on October 29, when the patient appeared to be entirely well and the spleen could no longer be felt, the cold agglutinin titer was still undiminished. However, a subsequent examination on December 17 revealed that the cold agglutinins had almost completely disappeared from the blood, only a trace of agglutination being present at a serum dilution of 1-8. The association of the serological phenomenon with the acute phase of the illness, and its disappearance following the specific treatment and recovery of the patient, would apparently indicate that the cold agglutinins developed as the result of the infection and were not merely a chance finding. This conclusion is borne out by the discovery of similar antibodies in high titer in the second case of leishmaniasis.

The reasons for the development of cold agglutinins and their significance in leishmaniasis are unknown, and the incidence of their occurrence in the disease is yet to be determined. This report is made with the hope that investigators more favorably situated may cast further light on these problems.

Summary. Cold agglutinins in high titers were demonstrated in the blood of two patients suffering from visceral leishmaniasis. In one of these patients, who was followed for several months, the agglutinins returned to within normal limits after recovery.

14855

Isolation from Fresh Human Serum of a Fraction which Inhibits Certain Serological Reactions.*

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The suppressive action of fresh sera, human or of other animal species, on immune precipitation as well as on bacterial agglutination (prezone phenomenon) has been reported in numerous publications.[1-6] Moreover, it has been general practice to heat sera prior to use in flocculation tests for syphilis, although the reason for this procedure has never been adequately explained.[7]

Two years ago, Major Charles R. Rein of the United States Army (Medical Corps), in a personal communication to the authors, reported that the addition of fresh normal human serum to heated syphilitic sera causes the inhibition of formation of aggregates in various flocculation tests. He also noted that heated normal serum does not show this inhibiting effect. At his suggestion, the present authors undertook a study of this phenomenon, and now report further evidence for the existence, and methods for the isolation, of a thermolabile fraction of human serum responsible for the inhibition of certain serological reactions.

The Inhibitory Effect of Whole Serum. The inhibitory action of fresh human serum was studied in the Kahn, Kline, Mazzini, and Boerner-Jones-Lukens precipitation tests, and was demonstrated in these tests with approximately 500 fresh syphilitic sera. The inhibition of the flocculation of the specific polysaccharide of the Type III pneumococcus and its homologous rabbit antiserum was also demonstrated.

Syphilitic sera were found to give negative reactions in the fresh state, and to be positive only after heating or standing for given periods of time dependent upon the temperature. Thus, at –35°C, these sera were found to be negative, even after one year, demonstrating that the inhibitor present in the serum could be preserved at this temperature. However, raw syphilitic sera stored at 3-4°C lost their inhibitory powers, and thus gave positive serological reactions, after several weeks. Further, as the temperature of storage increased, the period of time necessary for the achievement of serological positivity decreased. Finally, the inhibitory factor in syphilitic sera was found to be destroyed in 10 to 30 minutes at 56°C. The inhibitory effect of fresh nonsyphilitic sera was found to be similarly labile at these conditions of temperature and ageing.

The addition of strong electrolytes such as the chlorides, bromides, and iodides of sodium, potassium, and ammonium, to final concentrations above 2%, caused a syphilitic serum to show a positive reaction with about the same intensity as that shown by the serum inactivated by heating at 56°C for 30 minutes. This observation is undoubtedly related to the rationale for the Kline,[8] Laughlen,[9] and Briceño Rossi[10] tests, all of which employ hypertonic salt solutions. The effect of salt on the inhibitor, in contrast to the heat effect, was shown to be reversible, inasmuch as the removal of the salt by dialysis, or dilution of it to isotonicity, caused the serum to react

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* Aided by a grant from the Commonwealth Fund.

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[7] Eagle, H., *The Laboratory Diagnosis of Syphilis*, C. V. Mosby Co., St. Louis, 1937.

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[9] Laughlen, G. F., *Can. Med. Assn. J.*, 1935, **33**, 179.

[10] Briceño Rossi, A. L., *Rev. de Sanidad y Asistencia social*, 1942, **7**, 333.

negatively once more.

Non-electrolytes such as sucrose, glucose, urea, and glycerol, used in concentrations as high as 5%, did not cause inactivation, reversible or irreversible, of the inhibitor in fresh serum.

The Separation of the Inhibitory Factor from Fresh Syphilitic Serum. Fifty ml of a clear syphilitic serum was enclosed in a Visking cellophane membrane (20/32, No. 213) and dialyzed with mechanical rotation at 1°C against 8 liters of a phosphate buffer of ionic strength 0.02 and pH 5.4. The buffer was prepared by adding 153 ml of 0.5 M KH_2PO_4 solution and 1.73 ml of 1 N KOH to sufficient distilled water to make 4 liters of solution.[11] After 48 hours of dialysis, the contents of the bag were centrifugalized in a brine-cooled centrifuge at 1°C at 2000 r.p.m. for 30 minutes, and the supernate (labeled "supernate A") poured off, neutralized with 1 N NaOH, and rendered isotonic with the requisite amount of 18% NaCl. The precipitate (labeled "precipitate A"), now sedimented, which had formed in the dialysis, was washed 3 times by suspension in and centrifugation with the phosphate buffer at pH 5.4. After the third washing, the precipitate was evenly distributed in 50 ml of the buffer, and 5 ml of this suspension was withdrawn into another tube. Both portions of the precipitate were then centrifugalized and the supernates discarded.

When tested in the Kline and Kahn tests, supernate A was found to be positive by itself, whereas 5 ml of supernate A combined with the sediment obtained from the 5 ml of the suspension of precipitate A, was found to be negative. It was also shown that the addition of precipitate A to a pneumococcus SSS III-specific antiserum system resulted in the inhibition of flocculation in the region of antibody excess.

The inhibitor substance was further separated from precipitate A by extraction of this latter fraction in twice its volume of a buffer of pH 6.6 prepared by dissolving 75 ml of 0.5 M KH_2PO_4 and 10.58 ml of 1N KOH in sufficient distilled water to make one liter of solution. The buffer, first cooled to 1°C, was mixed with precipitate A, and the mixture allowed to stand in a water bath at this temperature for 30 minutes. At the end of this time it was centrifugalized at 1°C, and the sedimented precipitate was washed twice with the same buffer. The precipitate was then dissolved in 1.8% NaCl to a volume equal to one-tenth of the volume of serum from which it had been prepared. This fraction, labeled "precipitate B," represented, as found in several preparations, from 0.6 to 1% of the total serum proteins, and usually contained, in addition to the inhibitory factor, a small amount of C'l of complement.[11] Occasionally, precipitate B was found to be entirely free from C'l activity.

In the preparation described above, syphilitic serum was used in order to demonstrate that the removal of the inhibitor causes the remaining portion of the serum to become serologically positive. However, it is emphasized that a similar procedure may be used for the separation of the inhibitor from normal (non-syphilitic) serum. Table I shows the inhibitory action of the several fractions prepared from normal serum.

Summary. From human serum (syphilitic and non-syphilitic) a fraction comprising from 0.6 to 1% of the total serum proteins, and containing an inhibitor for certain serological reactions such as the Kline and Kahn tests, has been isolated by means of chemical fractionation with phosphate buffers. This inhibitory substance as it occurs in serum, may be reversibly inactivated by hypertonic solutions of electrolytes, and irreversibly by heat.

TABLE I.
The Inhibitory Action on the Kline Test of Fresh Human Serum and Several of Its Fractions.

0.3 ml of heated syphilitic serum mixed with 0.15 ml of:*	Kline test
Heated normal serum	++++
Fresh " "	—
Precipitate A†	— or ±
" B‡	— or ±

* All fractions used in 1:1 dilution with respect to whole serum.

† Comprising about 5% of the total serum proteins.

‡ Comprising from 0.6 to 1% of the total serum proteins.

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[11] Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, **47**, 181.

14856

Water, Electrolyte and Nitrogen Content of Human Skin.

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This study on the distribution of water, nitrogen, and electrolytes in human skin (corium and epidermis) was made for the purpose of defining normal values which will serve as a basis of comparison for later investigations on abnormal skin. The report gives data on a group of patients from which skin was obtained during the course of surgery: fifteen specimens taken during radical mastectomies and three during leg amputations.

The electrolyte content of human skin has not been comprehensively studied in the past, and the limited data in the literature are unsatisfactory because the values are not expressed on a fat-free basis. The available analytical data on human skin are summarized in Table I.

Methods. Eighteen tissues were available for analysis: fifteen from the breast and three from the lower extremities. The tissues were removed immediately from an amputated part and placed in a glass-stoppered weighing bottle. The skins were taken as far from any pathological condition as possible. The preparation of the skin for analysis was as follows: Each skin was quickly placed on a tile and trimmed to remove any muscle and as much free fat as possible. It was wiped with gauze, cut into small strips approximately 0.5 x 2 mm and placed in a weighed glass-stoppered weighing bottle. After being weighed, the bottle was placed in a 100° oven and dried to constant weight.

The dried skin was then extracted for neutral fat[6] and the strips of skin were transferred quantitatively to a special apparatus[6-8] and pulverized. The smashed material was put through a No. 20 copper sieve after which it was transferred to an agate mortar and ground to a homogeneous mixture. This mixture was returned to a glass-stoppered weighing bottle and kept in a desiccator over sulfuric acid. Before each aliquot was taken from the bottle the mixture was stirred thoroughly with a stainless steel spatula.

The chemical methods used in the analyses were the same as those employed in our previous work.[9] The following determinations were made: water, chloride, sodium, potassium, calcium, magnesium, total nitrogen, and collagen nitrogen.

Results and Discussion. Values for Normal Skin. The analytical data of the skins from 18 patients are given in Table II, together with the mean values with standard deviations. Since skin is so complex in structure and since the samples of tissue analyzed comprised both the corium and epidermis layer, it was not surprising that the results were not as constant as have been found for other tissues. For example, the total water content on 15 of the 18 skins varied between 69.0 and 74.0%. It should be borne in mind that the skins were removed from different parts of the body that had been amputated. In our previous study on skins removed from the dog, the skins had been removed directly from the same location on the body, using a uniform technic in every animal. The difference in technic for the removal of the skins might account in part for the larger standard deviations for the sodium, chloride, and water in the group of human

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[1] Brown, H., *J. Biol. Chem.*, 1926, **68**, 729.

[2] Nathan, E., and Stern, F., *Dermat. Z.*, 1928, **54**, 14.

[3] Volk, R., and Fantl, P., *Dermatologica*, 1939, **79**, 91.

[4] Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, 1941, **139**, 795.

[5] Cornbleet, T., Ingraham, R. C., and Schorr, H. C., *Arch. Dermat. and Syphilol.*, 1942, **46**, 833.

[6] Eichelberger, L., and Bibler, W. G., *J. Biol. Chem.*, 1940, **132**, 645.

[7] Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., *J. Biol. Chem.*, 1934, **104**, 149.

[8] Eichelberger, L., *J. Biol. Chem.*, 1941, **138**, 583.

[9] Eichelberger, L., Eisele, C. W., and Wetzler, D., *J. Biol. Chem.*, 1943, **151**, 177.

TABLE I.
Analytical Data in the Literature.
Values were calculated in units per 100 g of dry skin.

Investigator	Source of tissue	Cl mM	Na mM	K mM	Ca mM	Mg mM	H_2O g	Collagen g
Brown, 1926[1]	necropsy		15.6	6.1	1.15	1.23		
Nathan *et al.*, 1928[2]	15 patients			6.4	0.61		250	
Volk, 1939[3]	24 patients, biopsy	24.7*					275*	
Lowry *et al.*, 1941[4]	2 patients							79
Cornbleet *et al.*, 1942[5]	biopsy		15.2	6.3	1.07			

* Values expressed in terms of fat-free solids.

TABLE II.
Water, Electrolyte, and Nitrogen Content of Human Skin.
The values are expressed in units per kilo of fat-free skin.

Patient unit No.	Source of skin	H_2O g	Cl mM	Na mM	K mM	Ca mM	Mg mM	Total N, g	Collagen N, g
x	Breast	725.5	73.5	88.2	15.41			44.3	35.4
155220	Thigh	740.5	76.1	96.2	18.60	3.75	2.06	40.65	29.35
281123	Breast	725.0	79.8	95.0	10.17	2.77	1.58	44.37	
78716	”	725.0	78.8	93.6	13.28	2.84	2.02	42.0	28.7
141323	”	695.5	74.6	85.8	10.58	2.43	2.50	49.3	41.9
285023	”	677.0	84.8	93.1	14.88	1.62	2.39	52.3	
290098	”	695.2	83.3	83.8	19.07	3.52	1.94	47.56	
206536	Leg	713.6	72.9	89.2	16.88				
y	Breast	708.1	84.2	93.7	23.46	3.04	2.66	45.2	31.1
238104	”	691.5	81.8	85.9	16.2	2.98	3.45	52.1	40.7
23792	”	741.9	82.8	102.8	16.8	2.72	1.79	42.1	28.7
326543	”	704.8	74.3	81.7	14.64	2.09	2.12	49.7	38.6
323121	Leg	728.9	90.1	108.9	20.80	3.24	2.25	44.9	30.0
45193	Breast	731.5	85.5	95.4	19.6	2.40	2.10	44.2	33.0
330802	”	706.0	76.6	88.6	17.6	2.50	1.82	48.2	38.8
244188	”	715.0	78.8	94.1	15.8	2.48	1.88	46.0	35.0
280907	”	756.0	84.2	112.0	16.6	2.23	1.91	38.6	28.3
335101	”	738.8	76.0	85.4	16.08	2.33	1.66	41.4	32.6
No. of determinations		18	18	18	18	16	16	17	14
Mean		717.7	79.9	93.0	16.47	2.68	2.13	45.5	33.7
σ		20.1	4.8	8.0	3.36	0.52	0.30	3.8	4.5
			Dog	Skin.[9]					
Mean		708.3	86.7	96.5	22.46	3.01	3.03	46.8	33.1
σ		20.1	2.5	4.2	2.7	0.52	0.37	3.7	3.0

σ = Standard deviation.

skins than in the dog skins.

It will be noted, however, that a number of similarities of analytical results did occur between human and dog skin, the most pronounced exception being potassium (Table II and IV). If the results are expressed in units per 100 g of fat-free skin solids (Table III), the similarities are more apparent, with the lower potassium values in human skin still prevailing. The total nitrogen content (45.5 g per kilo, ±3.8 g) and the collagen nitrogen content (33.7 g per kilo, ±4.5 g) of the human skin were the same within experimental limits of error, as that found in dog. The 33.7 g of collagen nitrogen represents 188 g (5.58 × 33.7) of collagen per kilo of fat-free skin and since the total solid matter of human skin was 282 g per kilo, the remaining 94 g must be allocated to the other proteins found in skin. Thus collagen represents two-thirds of the solid matter of skin.

From the collagen nitrogen content the connective tissue nitrogen and therefrom the connective tissue weight were calculated by the method that Manery *et al.*[10] used for muscle; that is, by assuming that the connective tissue of skin corresponds to tendon and that the

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[10] Manery, J. F., Danielson, I. S., and Hastings, A. B., *J. Biol. Chem.*, 1938, **124**, 359.

TABLE III.
Skin Analyses of Human and Dog Skin.
Values are expressed in units per 100 g of fat-free solid.

	H_2O g	Cl mM	Na mM	K mM	Ca mM	Mg mM	Total N. g	Collagen N, g
Human skin (18 subjects)	254	28.3	32.9	5.83	0.95	0.75	16.1	11.9
σ	23	2.6	4.5	1.27	0.22	0.12	0.2	1.1
Dog skin[9] (10 subjects)	243	29.7	33.1	7.71	1.03	1.04	16.0	11.3
σ	24	2.3	3.2	1.14	0.20	0.08	0.3	0.8

σ = Standard deviation.

TABLE IV.
Estimated Weight of Connective Tissue in Human Skin with Calculated Concentrations of Water, Chloride, Sodium, and Potassium in This Tissue.
The values are expressed in units per kilo of fat-free skin.

Exper. No.	Collagen N g	Connective tissue N g	Connective tissue weight, g	Connective tissue H_2O g	H_2O* g	Connective tissue Cl mM	Cl* Mm	Connective tissue Na mM	Na* mM	Connective tissue K mM	K* mM
x	35.4	38.5	572	347	378	44.7	28.8	53.4	34.8	3.41	11.92
155220	29.4	32.0	476	289	452	37.2	38.9	44.5	51.7	2.90	15.70
78716	28.7	31.3	455	276	449	36.4	42.4	43.4	50.2	2.78	10.50
141323	41.9	45.6	677	411	285	52.9	21.7	63.2	22.6	4.13	6.45
y	31.1	33.9	504	306	402	39.4	44.8	47.1	46.6	3.07	20.39
238104	40.7	44.3	658	399	292	51.5	30.3	61.5	24.4	4.01	12.19
23792	28.7	31.3	465	282	460	36.4	46.4	43.4	59.4	2.84	13.96
326543	38.6	42.0	624	378	326	48.8	25.5	58.2	23.5	3.81	10.83
323121	30.0	32.7	486	295	434	37.8	52.3	45.4	63.5	2.96	17.84
45193	33.0	35.9	533	323	408	41.7	43.8	49.7	45.7	3.25	16.35
330802	38.8	42.3	628	381	325	49.1	27.5	58.6	30.0	3.83	13.77
244188	35.0	38.1	566	343	372	44.3	34.5	52.8	41.3	3.45	12.35
280907	28.3	30.8	458	278	478	35.8	48.4	42.9	69.1	2.79	13.81
335101	32.6	35.5	528	320	419	41.3	34.7	49.3	36.1	3.22	12.86
Mean	33.7	36.7	546	330	391	42.7	37.1	51.0	42.8	3.32	13.50
σ	4.5	4.9	73	45	61	5.7	9.1	6.8	14.5	0.45	3.29
					Dog Skin.[9]						
Mean	33.1	34.7	540	328	381	42.5	44.4	48.9	46.5	3.29	19.14
σ	3.0	3.1	46	28	36	3.6	4.5	4.5	7.4	0.89	2.78

*Total skin concentrations minus connective tissue concentration.
σ = Standard deviation.

ratio of collagen nitrogen to total nitrogen is the same in all connective tissue as it is in tendon. Employing the assumption that the ratio of collagen nitrogen to total nitrogen in the tendons of man is the same as that found in the tendons of dog, the data from dog tendons were used to calculate the weight of the connective tissue in the human skin: average total nitrogen content, 67.3 g; collagen nitrogen, 61.8 g; total water 606.2 g and chloride 78.2 mM per kilo of fat-free tendon.[11]

From the data in Table II the weight of the connective tissue of skin was estimated; and the amounts of water, chloride, sodium, and potassium identified with this connective tissue were calculated using the above tendon data along with the sodium and potassium data given by Muntwyler *et al.*[12] The results of these calculations are presented in Table IV. The water, chloride, sodium, and potassium in excess of that accounted for by the connective tissue should represent the amounts associated with the remaining part of the corium and the entire epidermis, since no collagen is found in the epidermis. In a kilo of skin, therefore, 546 g may be allocated to the connective tissue and 454 g to the cells of the skin and their surrounding ultrafiltrate. The numerical amount of water, chloride, and sodium that can be assigned to the ultrafiltrate volume cannot be satisfactorily estimated on the basis of what we know now; nevertheless, the low concentration of potassium (13.5 mM) suggests that the amount of the intracellular phase of the skin is small; and if magnesium is ascribed to the intracellular fluids of a tissue, the low concentration found in the skin (2.13 mM) should further corroborate this small intracellular phase.

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[11] Eichelberger, L., and Brown, J. D., *Proc. Am. Soc. Biol. Chem.*, 1944, **3**, 56.

[12] Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H., *J. Biol. Chem.*, 1940, **134**, 389.

Although the total water of the extracellular phase cannot be calculated at this time, the calculated 330 g of water identified with the 546 g of connective tissue per kilo of fat-free skin demonstrates that the volume of available water is considerably larger in the skin than any other tissue of the body. This finding corresponds to the results obtained by Flemister[13] for his work on mice.

Summary. Total water, nitrogen, collagen nitrogen, and electrolyte concentrations were determined in human skin which was removed at once from amputated parts during the course of surgery. The total skin (corium plus epidermis) gave the mean average results which are expressed as units per kilo of fat-free skin: total water, 717.7 ± 20.1 g; chloride, 79.9 ± 4.8 mM; sodium, 93.0 ± 8.0 mM; potassium, 16.47 ± 3.36 mM; calcium, 2.68 ± 0.52 mM; magnesium, 2.13 ± 0.30 mM; total nitrogen, 45.5 ± 3.8 g; and collagen nitrogen, 33.7 ± 4.5 g.

By applying methods now accepted as valid for calculating the weight of connective tissue in a tissue, the weight of connective tissue in human skin was estimated to be 546 ± 73 g per kilo of fat-free skin. The calculated 330 ± 45 g of water identified with this amount of connective tissue demonstrates that the volume of available water is considerably larger in skin than in any other tissue of the body. The skin may be regarded as one of the most important water-storage organs of the body.

We wish to express our appreciation to various members of the Department of Surgery for making the tissues used in this study available to us.

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[13] Flemister, L. J., *Am. J. Physiol.*, 1942, **135**, 430.

14857

Effect of Penicillin on Experimental Staphylococcus Osteomyelitis in Rats.*

J. EMERSON KEMPF AND J. ARTHUR HERRICK. (Introduced by Malcolm H. Soule.)
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Experimental staphylococcal infections in animals which simulate natural occurring infections in man are difficult to produce. In order to evaluate the recently introduced chemotherapeutic agents it would be desirable to have access to readily reproduceable *in vivo* procedures. The purpose of this study was to develop methods for the production of chronic staphylococcal infections in mice and determine the value of penicillin in the treatment of such infections.

With economy of penicillin in mind, several animals were considered, and on investigation the rat was found to be suitable for the purpose. The production of osteomyelitis in this animal did, however, present a major problem which was quite distinct from that of treatment. Because of the nature of the problem and the extent of the experimental work involved, it is deemed desirable to report this study as a separate publication.

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* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

Experimental Work. The method of producing the infections finally employed was as follows: Albino rats, Wistar strain, weighing 50 to 100 g, were anesthetized with nembutal, and a small hole (about 2 mm diameter) drilled into the bone from the medial surface at the proximal end of the tibia. With the aid of curved forceps, a pledget of cotton soaked with a 24-hour broth culture of *Staph. aureus* was inserted into the bone marrow. The strain of organism used was originally isolated from a patient with chronic osteomyelitis of hematogenous origin. The skin was then sutured with No. 50 white cotton thread.

After a period of from 21 to 26 days, the surviving animals were again anesthetized, the cotton pledgets removed and the character and extent of the lesions observed. Cultures were made by inoculating proteose peptone broth with material from deep in the involved area, and incubated at 37°C for 24-48 hours. When growth occurred, smears were made and stained by Gram's method.

A total of 80 animals was inoculated; 70% developed osteomyelitis, 19% died during the developmental period, and in 11% the initial lesions healed uneventfully. To be sure that the desired infection had been produced, material from 14 of the animals was examined histologically[†] and found to be acute or chronic osteomyelitis in each instance.

Two experiments were performed to test the effect of penicillin. The treatment was instigated promptly following the establishment of typical infections. In the first experiment, each of 14 rats was given 600 units of the sodium salt of penicillin subcutaneously every 2 hours for a period of 9 days, a total of 64,800 units. Fifteen animals received no drug and served as a control. Two hours after the final injection of penicillin, samples of blood were obtained by cardiac puncture and the penicillin content determined by the Oxford cup method as described by Abraham *et al.*[1] At this time the blood level was found to be consistently less than 0.1 unit per ml. Gross examinations revealed that of the 14 treated animals, 5 had lesions which had decreased in size and appeared to be healing, 3 showed no change, and 6 had apparently recovered, *i.e.*, the lesions had either completely healed or reduced in size and contained sterile pus. In the control group the lesions had progressed in every instance but one in which spontaneous recovery had apparently taken place. Cultural material from all animals in which recovery had not occurred was positive for staphylococci.

It appeared that the penicillin had exerted a beneficial effect. Since the blood content of penicillin dropped to a very low level between injections it was thought that larger amounts given at more frequent intervals might produce more favorable results.

In a second experiment the methods were essentially the same except that the dosage of the drug was increased. Each of 13 animals received 900 units of penicillin every hour, instead of 600 units every 2 hours, and, therefore, a total of 194,400 units during the 9 days. One hour after the final injection the blood level was determined and again found to be consistently less than 0.1 unit per ml. Cultures of this blood showed the presence of staphylococci in all animals including the controls. On macroscopical examination the lesions in 6 of the treated rats had healed, in 3 there was no suggestion of change, and in 4 there was a more extensive involvement. On culturing material from the original areas of inoculation, the six in which healing had occurred gave no growth, even though blood cultures from these animals were positive for staphylococci. The specimens from the other 7 treated animals all gave positive cultures of staphylococci. Of the 10 control animals in this experiment, none showed any evidence of recovery and pus from all yielded positive cultures of staphylococci.

Discussion and Conclusions. The data of the above experiments indicate that penicillin does exert a beneficial effect on experimental osteomyelitis in rats. The fact that the blood level of the drug drops very rapidly following the injection of such heavy doses led to efforts to maintain higher levels by simultaneously injecting other materials designed to reduce kidney excretion or to decrease the rate of absorption. No satisfactory method was found. It is of interest to note, however, that Romansky and Rittman[2] have recently reported such an accomplishment by mixing penicillin with beeswax and peanut oil. Although they report no development of sensitization to these accessory substances, this has not been the experience of others. The fact that the 900 units per hour produced no better results than 600 units every 2 hours, plus the low blood levels found following the final injections, indicates that if some method of maintaining a nearly constant level were used, the level could be rather low and still produce good results. Since staphylococci were consistently found in the blood, even after the lesions had healed, it seems that the treatment should be continued for a greater period of time.

[†] Kindly examined by Dr. Carl V. Weller of the Department of Pathology.

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

These results indicate that penicillin has definite value as a systemic therapeutic agent for staphylococcus osteomyelitis in rats; and further, that the rat is a suitable animal for studying the course of experimental osteomyelitis, and the evaluation of therapy in this disease.

Summary. Osteomyelitis was produced experimentally in white rats by inoculating the bone marrow of the tibia with *Staph. aureus.* About 21 days were allowed for the development of typical lesions. One group of 14 animals was treated by the subcutaneous injection of 600 units of penicillin every 2 hours for 9 days. Six of these apparently recovered during the course of the treatment. In a second experiment 13 animals were each given 900 units of the drug every hour for an equal period of time. Again 6 of the lesions were found to be healed at the termination of the injections. Of a total of 25 untreated controls only one animal showed evidence of recovery. It is concluded that penicillin has value as a systemic therapeutic agent in the treatment of the experimental disease, and that the rat is a suitable animal for the study of the course of experimental osteomyelitis and the evaluation of therapy.

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[2] Romansky, M. J., and Rittman, G. E., *Science,* 1944, **100**, 196.

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